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Intra-amniotic IL-1β induces fetal inflammation in rhesus monkeys and alters the regulatory T cell/IL-17 balance

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Abstract

Very low birth weight preterm newborns are susceptible to the development of debilitating inflammatory diseases, many of which are associated with chorioamnionitis. To define the effects of chorioamnionitis on the fetal immune system, IL-1β was administered intra-amniotically at ~80% gestation in rhesus monkeys. IL-1β caused histological chorioamnionitis as well as lung inflammation (infiltration of neutrophils or monocytes in the fetal airways). There were large increases of multiple pro-inflammatory cytokine mRNAs in the lungs 24 h post-administration, which remained elevated relative to controls at 72 h. Intra-amniotic IL-1β also induced sustained expression of the surfactant proteins in the lungs. Importantly, IL-1β significantly altered the balance between inflammatory and regulatory T cells (Treg cells). Twenty-four h after IL-1β injection, the frequency of $CD3^+CD4^+FOXP3^+$ T cells was decreased in lymphoid organs. In contrast, IL-17A–producing cells ($CD3^+CD4^+$, $CD3^+CD4^-$, and $CD3^-CD4^-$ subsets) were increased in lymphoid organs. The frequency of IFN-γ-expressing cells did not change. In this model of a single exposure to an inflammatory trigger, $CD3^+CD4^+FOXP3^+$ cells rebounded quickly and their frequency was increased at 72 h compared to controls. IL-17 expression was also transient. Interestingly, the T cell profile alteration was confined to the lymphoid organs and not to circulating fetal T cells. Together, these results suggest the chorioamnionitis-induced IL-1/IL-17 axis is involved in the severe inflammation that can develop in preterm newborns. Boosting Treg cells and/or controlling IL-17 may provide a means to ameliorate these abnormalities.

Introduction

Very preterm newborns frequently develop severe inflammatory diseases affecting multiple organs, including Bronchopulmonary Dysplasia, Necrotizing Enterocolitis (NEC), and postnatal sepsis (1). The connection between fetal inflammation and other morbidities of the premature infant, such as retinopathy of prematurity and cerebral palsy, are also of concern (2, 3). Although the origins of these pathologies are likely multifactorial, they are frequently

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associated with chorioamnionitis (4). Fetal inflammation has been assessed in clinical studies by measuring cytokine concentrations in amniotic fluid, neonatal plasma, and gastric and tracheal aspirates (5–7). Elevated levels of cytokines such as IL-6, IL-8, and TNF-α have all been associated with chorioamnionitis (5, 8–12). Intra-amniotic injection of live organisms in the macaque induced IL-1 and caused preterm labor (13, 14). We previously showed in fetal sheep that chorioamnionitis induced with the intra-amniotic injection of LPS or IL-1 resulted in inflammation, particularly of the fetal lung, gut, skin, and chorioamnion (15–17). IL-1 was central to this inflammation as blockade of IL-1 signaling in the amniotic compartment with a recombinant IL-1 receptor antagonist (IL-1RA)[2] largely inhibited the fetal lung and systemic inflammation caused by intra-amniotic LPS (18).

IL-1β has profound effects on the immune system, inducing chemokine and IL-6 production, which are particularly sensitive to IL-1β (reviewed in (19)). Importantly, IL-1 appears essential to the generation of the Th17 response, given that T cells from mice deficient in IL-1RI fail to express IL-17 upon antigen challenge (20). Therefore, we hypothesized that infection *in utero* would induce an inflammatory cascade that both can cause preterm labor and activate the fetal immune system. A relevant observation in the fetal sheep chorioamnionitis model was a decrease in the frequency of Treg cells in the gut and thymus (16, 21, 22). However, detailed studies are impractical in the sheep, due to the lack of reagents to interrogate the immune system. The rhesus macaque model offers an attractive alternative to evaluate immune modulation by chorioamnionitis because of the availability of many cross-reacting Ab and the high degree of similarity in the ontogeny of the immune system in rhesus macaques and humans. Indeed, by the second trimester of gestation, the lymphoid tissues of the rhesus monkey fetus have a complete repertoire of appropriately organized antigen-presenting cells, T cells, and B cells (23), similar to human fetuses (24). In contrast, development of lymphoid tissues is delayed in rodents (25). TLR and inflammasome systems are also conserved between non-human primates and humans (26, 27). Furthermore, many aspects of reproductive biology are very similar when comparing the rhesus macaque and humans (28, 29).

Novy and colleagues showed that intra-amniotic injection of IL-1β to the fetal macaques induced chorioamnionitis and preterm labor (30–33). However, these studies did not explore fetal tissues in detail or immune responses. Therefore, we used an intraamniotic exposure to IL-1β in fetal macaques to define the effects of chorioamnionitis on the fetal immune system.

Materials and Methods

Animals and sample collection

All animal procedures conformed to the requirements of the Animal Welfare Act and protocols were approved prior to implementation by the Institutional Animal Care and Use Committee at the University of California, Davis. Normally cycling, adult female rhesus monkeys (*Macaca mulatta*) (N=17) with a history of prior pregnancy were bred and identified as pregnant, using established methods (28). Activities related to animal care were performed as per standard California National Primate Research Center operating procedures. Animals were randomized to receive either 10 μg human recombinant IL-1β (Peprotech, Rockyhill, NJ) in 1 ml sterile saline or 1 ml sterile saline by ultrasound guided intra-amniotic injection at 130±2 days gestational age, which is approximately 80% of term gestation (term: 165±10 days). The dose of 10 μg was chosen based on previous work done

[2]Abbreviations:
BAL: Bronchoalveolar lavage; HIF-1α:Hypoxia-Inducible Factor 1; IL-1RA: IL-1 receptor antagonist; LN: lymph nodes; NEC: Necrotizing Enterocolitis; Treg cell: regulatory T cells.

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in macaques (30, 33). Furthermore, chorioamnionitis and fetal inflammation is induced by 100 μg IL-1 in fetal sheep, which are ~10 times larger than fetal macaques (16). Of note, high levels of IL-1β have been measured in amniotic fluid (34), but the relative exposure of the fetal compartment to IL-1 in human remains unclear.

Hysterotomies were performed and the gestational sac removed aseptically 24 or 72 h post-administration of IL-1β (Table I). Fetuses were euthanized with an overdose of pentobarbital then peripheral blood and tissues (spleen, mesenteric and mediastinal lymph nodes (LN), lung lobes, amniotic fluid) were collected. Broncho-alveolar lavage (BAL) was recovered by inflating the left lung to total lung capacity with normal saline followed by fluid withdrawal. The procedure was repeated 3 times, the fluids were pooled and used for cell counts and protein analysis.

Cell isolation and culture

Single-cell suspensions from spleen, mesenteric and mediastinal LN were prepared following tissue collection. Each LN was dissected and cells were mechanically detached from the surrounding membrane using a scalpel and fine tweezers. Spleen was diced and dissociated into a homogenous cell suspension using a pestle. Cell suspensions were passed through 70 μm cell strainers, washed in culture media (RPMI 1640) containing 10% FCS, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 2 mmol/l glutamine. Using approximately 10 ml of heparinized fetal blood, PBMCs were isolated using Ficoll-Hypaque (GE Healthcare, UK) gradient centrifugation within 3 h of collection. Red blood cell lysis was performed as needed, using ammonium chloride/potassium carbonate/EDTA (Lonza BioWhitakker, Pittsburgh, PA). Cells were rested overnight at 37°C and 5% CO2. After overnight culture, viability was consistently above 85% (trypan blue exclusion test). For cytokine measurements, cells were stimulated with 50 ng/ml PMA (Sigma-Aldrich, Saint Louis, MO) and 750 ng/ml ionomycin (Calbiochem, San Diego, CA) for 5 h with 10 μg/ml brefeldin A (Sigma-Aldrich) and 1 μl/ml monensin 1000X (eBioscience, San Diego, CA) added for the last 4 h.

Flow Cytometry

The following Ab clones were used: SP34-2 (anti-CD3); L200 (anti-CD4); M-A251 (anti-CD25); PCH101 (anti-FOXP3); B56 (Ki67); BNI3 (anti-CTLA-4), B27 (anti-IFN-γ) and 64CAP17 (anti-IL-17A). Cells were treated with 20 μg/ml of human IgG to block FcR, stained for surface markers for 30 min at 4°C in PBS, washed, and fixed in 1% paraformaldehyde. Intracellular staining for FOXP3 was performed using the eBioscience reagents according to manufacturer's instructions. A negative biological population for FOXP3 ($CD3^{-}CD4^{-}$ cells) was used as reference to establish the cut-off for $FOXP3^{+}$ cells in $CD3^{+}CD4^{+}$ T cells, as previously described (35, 36). Intracellular staining for cytokines (IL-17, IFN-γ) was performed using Cytofix/Cytoperm (BD Bioscience) according to the manufacturer's instructions. All Ab were titrated for optimal detection of positive populations and mean fluorescent intensity. At least 250,000 events were recorded for each sample. Doublets were excluded on the basis of scatter properties and dead cells were excluded using LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen). Data were analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

Immunohistochemistry

Immunostaining protocols used lung paraffin sections (5 μm) from 10% formalin-fixed tissues that were deparaffinized and rehydrated using a series of xylene and alcohol washes. Antigen retrieval was performed using either citrate buffer (pH 6.0) or 1 mM EDTA buffer (pH 8.0). Tissues were pre-treated with 3% hydrogen peroxide to inactivate endogenous peroxidases. The sections were incubated with either anti-human CD3 (Dakocytomation,

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Glostrup, Denmark, 1:50), CD4 (Leica Biosystems, Nussloch, Germany, 1:50), CD56 (AbD Serotec, Kidlington, UK, 1:100), CD68 (Dakocytomation, 1:250), MPO (Cell Marque, Rockland, CA, 1:200) in 10% normal goat serum at 4°C overnight, followed by biotin-labeled secondary antibody. Immunostaining was visualized using Vectastain ABC Peroxidase Elite kit to amplify the antigen-antibody complexes (Vector Laboratories). The antigen staining was further enhanced with nickel-diaminobenzidine (DAB), followed by the Tris-cobalt, and the nuclei were counterstained with nuclear fast red. In addition, hematoxylin and eosin (H&E) staining was performed for lung tissue and chorioamnion. The severity of chorioamnionitis was graded using Redline's criteria (37).

RNA Isolation, cDNA generation, and Quantitative RT-PCR (qPCR)

Total RNA was isolated from the fetal right lung using TRIzol (Invitrogen, USA). The quality and yield of the RNA was assessed with a Nanodrop fluorospectrophotometer (Nanodrop, Wilmington, DE). One μg of total RNA was used to synthesize cDNA using Verso cDNA kit (Thermo-scientific) following the manufacturer's protocol. Quantitative RT-PCR was carried out with the TaqMan® gene expression Master Mix and Rhesus specific TaqMan gene expression assays, performed in a 7300 Real Time PCR system (Applied Biosystems, Carlsbad, CA). Cycling conditions consisted of a 2 min incubation at 50°C, followed by a 10 min incubation at 95°C, followed by 40 cycles of alternating temperatures of 95°C for 15 sec and 60°C for 1 min. The Eukaryotic 18S rRNA (Applied Biosystems) was used as an endogenous control for normalization of the target RNAs and a sample from the control group was used as a calibrator.

Cytokine and cortisol measurement

Cytokine/chemokine concentrations were determined by Luminex using Non-Human Primate Multiplex kits (Millipore, Billerica, MA) according to the manufacturer's protocol. Concentrations were calculated from standard curves using recombinant proteins and are expressed in pg/ml. Cortisol was measured after extraction of plasma with ethyl ether using an ELISA assay as per manufacturer's instructions (Oxford Biomedical Research, Rochester Hills, MI). This test operates on the basis of competition between the enzyme conjugate and the cortisol in plasma for a limited number of binding sites on the antibody coated plate.

Statistical analyses

Parametric or non-parametric tests were used depending on the normality of data distribution. ANOVA or Kruskal-Wallis tests were first used to determine whether the 3 groups were significantly different, and followed by one-to-one group comparisons by t-tests or Mann-Whitney tests. Results were considered significantly different for p values <0.05. However, due to the limited number of samples per group, trends are reported (p between 0.05 and 0.15).

Results

Intra-amniotic IL-1β induces chorioamnionitis

The intra-amniotic injection of IL-1β or saline was performed in multiparous macaques of similar weight and age (Table I). Mean fetal weights at tissue collection were not different between groups. Confirming previous results with this model (33), IL-1β caused histological chorioamnionitis in all animals, which was characterized as grade 1 and stage 2 at 24 h, and as grade/stage 1 or 2 at 72 h (Fig. 1). Histological chorioamnionitis was not present in any of the control animals. T cell numbers in the decidua were not altered by intra-amniotic IL-1β (mean + SE of T cells/high power field were the following: 68.3 + 28.3; 66.9 + 8.4 and 80.2 + 7.1 in controls, IL-1 24h and IL-1 72h respectively; p=0.55, Kruskal-Wallis test).

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Intra-amniotic IL-1β induces lung inflammation and maturation

Control fetuses had no neutrophils or monocytes within the fetal airways. In contrast, intra-amniotic IL-1β increased neutrophils and monocytes in the BAL at 24 h, and inflammatory cells remained high at 72 h (Fig. 2A). IL-1β also induced indicators of lung maturation as indicated by the sustained increased expression of surfactant protein (A, B, C, and D) mRNAs in the fetal lungs (Fig. 3). We measured plasma cortisol levels since glucocorticoids are given routinely to induce lung maturation in the preterm fetus (1). Control fetuses had low plasma cortisol levels (mean±SE: 3.7±0.2 μg/dL). Upon exposure to intra-amniotic IL-1β, the plasma cortisol increased significantly to 5.8±0.3 μg/dL at 24 h ($p<0.01$ vs. controls) with a decrease to 4.7±0.9 μg/dL at 72 h.

Intra-amniotic IL-1β also induced large increases in multiple pro-inflammatory cytokine mRNAs in the fetal lungs (Fig. 2, panels B–G). Indeed, the expression of IL-1β, IL-6, IL-8, and TNF-α mRNAs were increased by >50-fold at 24 h compared to controls. MCP-1 was also increased significantly, but to a lower value. These pro-inflammatory cytokines remained significantly higher than control values at 72 h, although they were decreased compared to the 24 h time point. The mRNA results were confirmed by measuring cytokine levels in the BAL (Fig. 4A). The pattern of increased pro-inflammatory cytokine proteins reflected the increased mRNA levels at 24 h, with levels decreasing at 72 h. IL-10 and IL-1RA also increased modestly, with the increase in IL-10 apparent at 72 h. In contrast, no significant increases were detected in IL-17, IL-4, IL-10, IFN-γ, or IL-2 mRNA expression in the lungs.

Double immunostaining for myeloperoxidase (MPO) and CD68 was used to characterize the lung infiltrating cells (Fig. 5 and Suppl Fig. 1). Consistent with the BAL data, the lungs of control fetuses contained very few neutrophils (expressing just MPO) or macrophages ($CD68^+$ or $CD68^+MPO^+$ cells). Upon exposure to intra-amniotic IL-1β, the neutrophilic (MPO^+CD68^-) population increased by more than 6-fold at 24 h and more than 4-fold at 72 h, and these cells consisted of approximately 70–80% myeloid cells in the IL-1β exposed animals. Activated macrophages ($CD68^+MPO^+$) were also significantly more abundant in the IL-1β exposed animals. These data suggest infiltration of fetal lung by predominantly neutrophils and some activated macrophages upon exposure to IL-1β. T cells were not detected in the lung or the BAL of control animals, and their numbers did not increase after IL-1β exposure (data not shown).

Intra-amniotic IL-1β alters ratios of Treg and IL-17-expressing cells in lymphoid organs

Intra-amniotic IL-1β did not change the overall proportion of $CD4^+CD3^+$ T cells in the mediastinal LN, mesenteric LN, spleen, or PBMCs at either time point (data not shown, all $p>0.20$, ANOVA). However, IL-1β altered the frequency of Treg cells (defined as percentage of $FOXP3^+$ cells in the $CD3^+CD4^+$ population) in the mediastinal LN, mesenteric LN, and spleen. Treg cell frequency at 24 h decreased by ~2-fold compared to controls (p values ranging from 0.06 to 0.09, Mann-Whitney tests, Fig. 6). In this model of single exposure to an inflammatory trigger, Treg cell frequency was increased at 72 h compared to 24 h, reaching levels generally higher than those in controls (Fig. 6).

In control fetuses, as expected, $CD3^+CD4^+FOXP3^+$ expressed high levels of CD25 and low levels of CD127 (Fig. 7A and B). They also expressed high levels of CD39 and CTLA-4, compared to their $FOXP3^-$ counterparts (Fig. 7A and B). Intra-amniotic IL-1β did not change expression of these markers in Treg cell or non-Treg cells (not shown). Interestingly, in control animals, Treg cells tended to express higher levels of the cell cycling marker Ki67 than non-Treg cells (Fig. 7A and B); IL-1β significantly increased the levels of Ki67 in Treg cells at 24 h, but not in non-Treg cells (Fig 7C).

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In contrast, 24 h after IL-1β injection, percentages of IL-17A$^+$ cells in the CD3$^+$CD4$^+$, CD3$^+$CD4$^-$, and CD3$^-$CD4$^-$ cells purified from the mediastinal LN, mesenteric LN, and spleen were increased compared to those in controls (Fig. 8 and Suppl. Fig. 2 for representative staining). Again, as described above for Treg cells, IL-17 induction was transient, as its expression at 72h was similar to that seen in controls animals (Fig. 8B). Baseline, unstimulated, IL-17 expression was similar between groups in all organs (data not shown, all $p>0.15$, Kruskal-Wallis tests). Interestingly, IL-1β did not significantly affect IFN-γ-expressing cells in any of the lymphoid tissues studied (Table II).

Immune changes induced by IL-1β in tissues and lungs were not detected in fetal blood

Because peripheral blood is the only compartment easily accessible in human premature newborns, we determined whether the changes occurring in tissues were also detectable in the blood. In contrast to the lymphoid organs, intra-amniotic IL-1β did not change Treg cell frequency in PBMCs (Fig. 6 panel D), nor did it induce IL-17 expression in PBMCs (Fig. 8 panel D). Similarly, levels of pro-inflammatory cytokines modestly increased in the blood, compared to the levels measured in the BAL (Table II). Plasma IL-6 significantly increased at 24 h in IL-1-treated animals (Fig. 4B), but the other pro-inflammatory cytokines that were increased in the fetal BAL of treated animals (IL-1β, IL-8, TNF-α, GM-CSF, MCP-1) did not simultaneously augment in fetal plasmas (data not shown). Interestingly, plasma levels of IL-1RA and IL-10 modestly increased (Fig. 4B), following a similar pattern as the BAL (Fig. 4A).

Discussion

Until recently, the chorioamnionitis associated infection/inflammation that initiates the events leading to preterm labor was thought to be an ascending colonization of the endometrium leading to a diffuse invasion of the chorion and amnion prior to dissemination to the amniotic fluid and the fetus (38). However, more recent pathological evaluations support a different model of focal colonization (infection) of the chorioamnion with dissemination into the amniotic fluid (and to the fetus) prior to the development of diffuse chorioamnionitis (39, 40). This distinction in the pathways to infection/inflammation of the fetal compartment is important as it changes the direction of pro-inflammatory signal for preterm labor from the uterus to the fetal compartment. Therefore, a simple model of intra-amniotic exposure may best reflect the focal membrane contamination that progresses to disseminated amniotic fluid infection followed by diffuse chorioamnion colonization prior to preterm labor (41, 42). Using such a model, acute chorioamnionitis caused fetal inflammatory responses and profound changes in T cell responses at 24 h, which reverted to normal by 72 h. Intra-amniotic IL-1β induced a sustained response in fetal tissues, as chorioamnionitis was more pronounced at 72 h than at 24 h, and lung cytokine expression remained increased at 72 h.

To our knowledge, our study provides the first experimental evidence that increased IL-17 responses could be induced in the fetus following chorioamnionitis. IL-1β and IL-6 are potent inducers of IL-17 (43, 44), and both cytokines were upregulated in the chorioamnion of IL-1- exposed macaques (data not shown). We also found significant up-regulation of these cytokines at 24 h in the lungs with lower expression at 72 h. Due to the short half-lives of cytokines, it is likely that the endogenously produced pro-inflammatory cytokines were responsible for the IL-17 induction. In contrast to its effect on IL-17, intra-amniotic IL-1β did not change IFN-γ expression by fetal cells. These data fit with a recent report indicating that CD4$^+$ T cells from extremely preterm infants have a low Th1 bias, but a significant Th17 bias, which was linked to their high baseline levels of RORγt and STAT3 mRNA (45). Interestingly, in our model, several cell populations expressed IL-17, including CD3$^+$CD4$^+$, CD3$^+$CD4$^-$, and CD3$^-$CD4$^-$ subsets. We did not further characterize these

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$CD3^{-}IL\text{-}17^{+}$ cells, and further analysis will be warranted to address this question. They may be NK cells or fetal lymphoid tissue inducers (LTi), which both lack CD3 and are known IL-17 producers (46). Alternatively, they may be T cells that internalized the TCR upon activation. Macrophages also produce IL-17 in some cases, but these $IL\text{-}17^{+}$ macrophages are in inflamed tissues or tumors, not in lymphoid organs (47–49). Neutrophils also produce IL-17 (50), but the $CD3^{-}CD4^{-}IL\text{-}17^{+}$ cells in the lymphoid organs did not have the distinct forward/scatter characteristics of neutrophils.

Taken together, our results suggest that the preterm nonhuman primate fetus has the capacity to mount an IL-17 response, although it was transient in this model using a single sterile cytokine as the inflammatory trigger. This experimental exposure would not be expected to induce IL-23, the most potent cytokine to stabilize the Th17 phenotype, as IL-23 production is mainly prompted by TLR-mediated stimulation of antigen-presenting cells (51). Neonatal mononuclear cells are high producers of IL-23, while being poor IL-12 producers (52–54). Of note, our recent results in a sheep model showed relatively long-lasting effects of LPS-induced inflammation on the fetal thymus, as upregulation of IL-17 mRNA and down-regulation of FOXP3 mRNA persisted several days after the exposure (55). However, we acknowledge that such acute model of inflammation does not fully reproduce human chorioamnionitis, which is often a chronic fetal exposure to inflammatory challenges. It will be important in the future to use the Rhesus macaque model to explore the fetal immune system in setting of placental colonization by Mycoplasma or Ureaplasma, which are frequently associated with preterm birth and poor outcomes (56).

Compared to controls, the frequency of $CD4^{+}FOXP3^{+}$ cells in all lymphoid organs decreased 24 h after IL-1 injection, while rebounding at 72 h. This pattern was reciprocal to the frequency of Th17 cells in the same lymphoid organs. These $CD4^{+}FOXP3^{+}$ are likely Treg cells, as they expressed significantly higher levels of Treg cell functional markers compared to their $FOXP3^{-}$ counterparts (Fig. 5), although suppression assays could not be performed to confirm these findings, due to limited cell yields. These results in a non-human primate model thus confirm our previous findings in fetal sheep, in which we showed that chorioamnionitis decreases Treg cell frequency in thymus and gut (16, 21, 22). Notably, a recent clinical study showed a significantly reduced proportion of Treg cells in the intestinal mucosa of premature infants with NEC compared to age-matched controls (57). Importantly, Treg cell proportions correlated with increased mucosal pro-inflammatory cytokine levels, and they were normalized in the healing phase, at the time of reanastomosis. Taken together, these results support the hypothesis that the strong inflammatory environment in the fetus inhibits Treg cell development or homeostasis, and that decreased Treg cell proportion may contribute to inflammatory disorders in premature babies. Mechanisms underlying the changed Treg cell/Th17 balance have not been elucidated, but they could involve the key metabolic sensor and regulator Hypoxia-Inducible Factor 1 (HIF-1α), which plays a key role in T cell fate determination (58, 59). Regulation of this pathway could include direct signaling of Treg cells by IL-1, as IL-1 can regulate HIF-1α levels (60) and activated Treg cells express high levels of IL-1R (61, 62). Interestingly, we found that Treg cells tended to express higher baseline levels of Ki67 than $CD4^{+}FOXP3^{-}$, a result in agreement with recent studies showing that Treg cell express high levels of cell cycle molecules *in vivo*, in both mice and humans (63–65). Ki67 expression in Treg cells, but not in non-Treg cells, was further increased 24 h after IL-1 compared to controls. This selective increase in cell cycle activation could explain the rapid rebound of Treg cells at 72 h. Further experiments will be necessary to clarify the signaling pathways triggered by chorioamnionitis that are able to affect T cell phenotypes in the fetus.

Notably, T cell changes in lymphoid tissues were not detected in fetal blood. This result is consistent with recent reports that the composition of T cell subsets is different in the

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lymphoid organs and the circulating blood of human fetuses, as evidenced by the fact that the frequency of memory $CD4^+$ T cells detectable in their lymphoid tissues (spleen, LN, gut-associated lymphoid tissues) is higher than are found in the fetal blood (66, 67). Similarly, several pro-inflammatory cytokines were greatly increased in the BAL but not in the fetal blood, with the exception of plasma IL-6 that reached levels indicative of fetal inflammatory response syndrome in the human (>11 pg/ml (8, 68)). Studies of immune modulation following *in utero* activation will not be easy to conduct in preterm humans, because the blood is the only immune compartment relatively accessible in this fragile population.

Another observation from this study was the increase in the mRNA for the four surfactant-associated proteins following IL-1β induced chorioamnionitis. There are two independent inducers of fetal lung maturation that are important clinically – maternal corticosteroid treatments and intrauterine exposure to inflammation (69). Bry et al. (70) first identified inflammation as an inducer of lung maturation following intra-amniotic injection of IL-1β in fetal rabbits. Lung maturation was detected as increased mRNA for surfactant proteins and improved pressure volume curves. Subsequently, intra-amniotic inflammation was demonstrated to consistently cause lung maturation in rodents and sheep (15, 71). To our knowledge, this is the first demonstration of lung maturation induced experimentally in a non-human primate. The magnitude of increase in mRNA for the surfactant proteins is comparable to that reported in other species (72). Based on our experience with fetal sheep (15), we did not measure pressure-volume curves or surfactant lipids, as they would not be expected to increase within 72 h. Although cortisol modestly increased after intra-amniotic IL-1β, it is unlikely that it mediated the lung maturation because plasma levels were about 10-fold lower than those reported in normal term humans (73).

Together, our data incriminate the chorioamnionitis-induced IL-1/IL-17 axis in the inflammation developing in very preterm newborns. The capacity of fetuses to mount a robust IL-17 response might constitute a double-edged sword, as it would afford them protection against infections by bacteria and fungus, but could also play a role in the development of the devastating inflammatory disorders often seen in these preterm babies. Our results also suggest that boosting Treg cells and/or controlling IL-17 may help control these pathologies. Furthermore, we demonstrate that the fetal rhesus macaque is a suitable model to study mechanisms of inflammation in human fetuses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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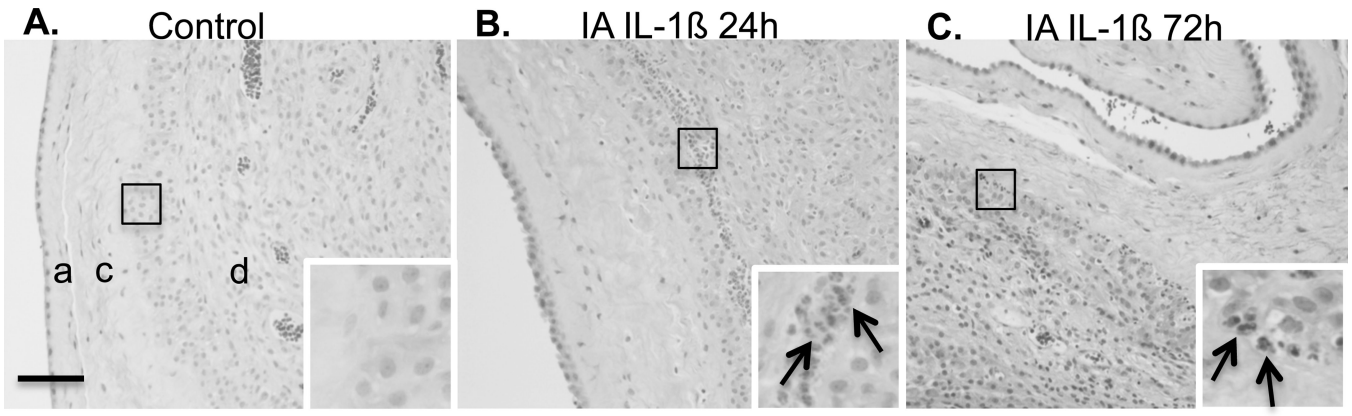

Fig. 1. Intra-amniotic IL-1β induces histological chorioamnionitis

Representative photomicrographs from H&E staining of chorioamnion and decidua are shown. (A) control; (B) IL-1 24 h; (C) IL-1 72 h exposure. A magnification of the region shown in each panel is shown as an insert. The arrows point to inflammatory cells observed after IL-1 exposure, which are absent in controls. (a=amnion, c=chorion, d=decidua; magnification bar represents 50 μm).

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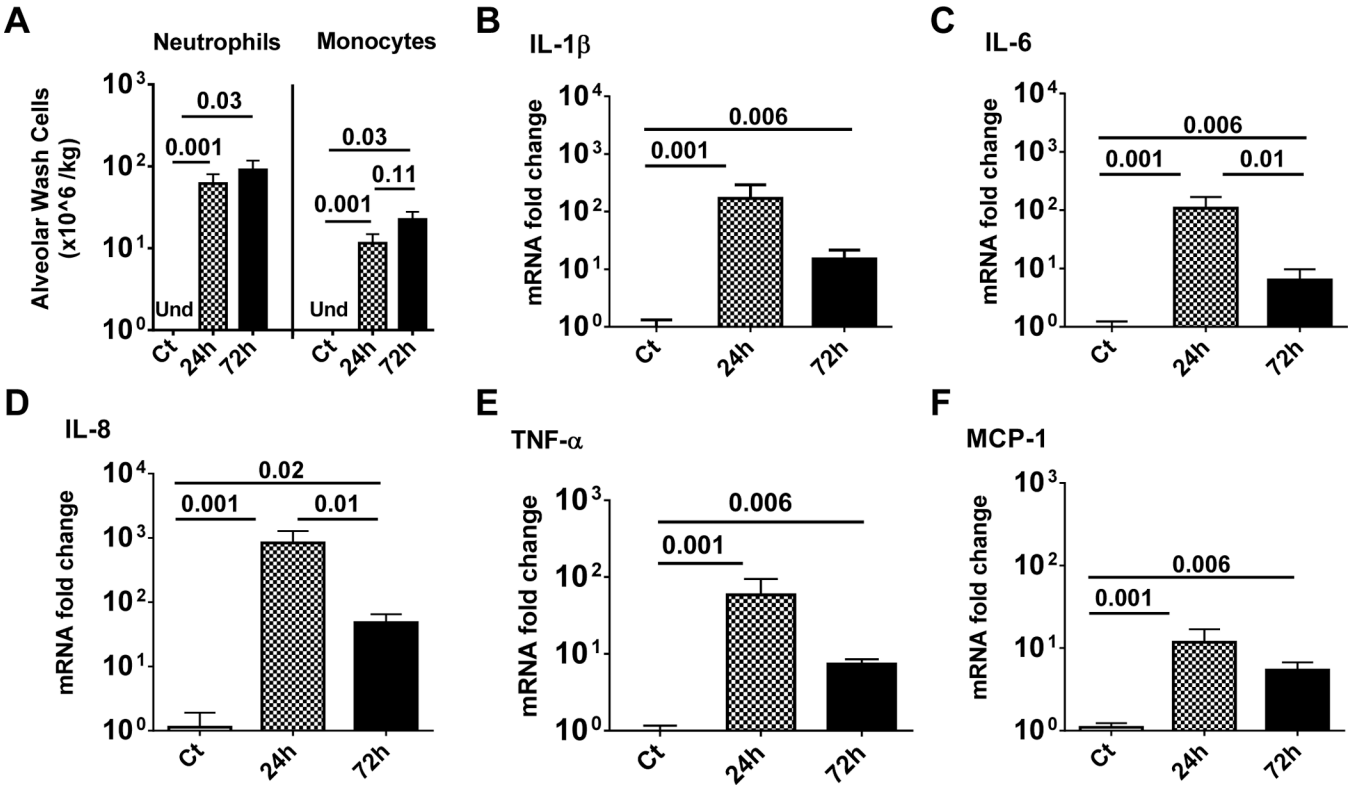

Fig. 2. Intra-amniotic IL-1β induces lung inflammation

In panel A, values are expressed as mean (SE) cells × 10^5/kg body weight (n=4–6/group). Cells were counted on cytospins of BAL stained with H&E (minimum of 200 cells). Cell numbers were calculated using the following formula: cells/ml x BAL volume (mL)/body weight (Kg). "Und" means undetectable. Significant *p* values or trends are shown (Mann-Whitney tests). In the other panels (panels B to F), values are expressed as mean (SE) fold change in cytokine RNA expression in the lung of each animal (n=4–6/group). Target RNAs were measured by quantitative RT-PCR, and normalized on the expression of eukaryotic 18S rRNA. A sample from the control group was used as a calibrator to calculate fold changes. Significant *p* values or trends are shown (Mann-Whitney tests).

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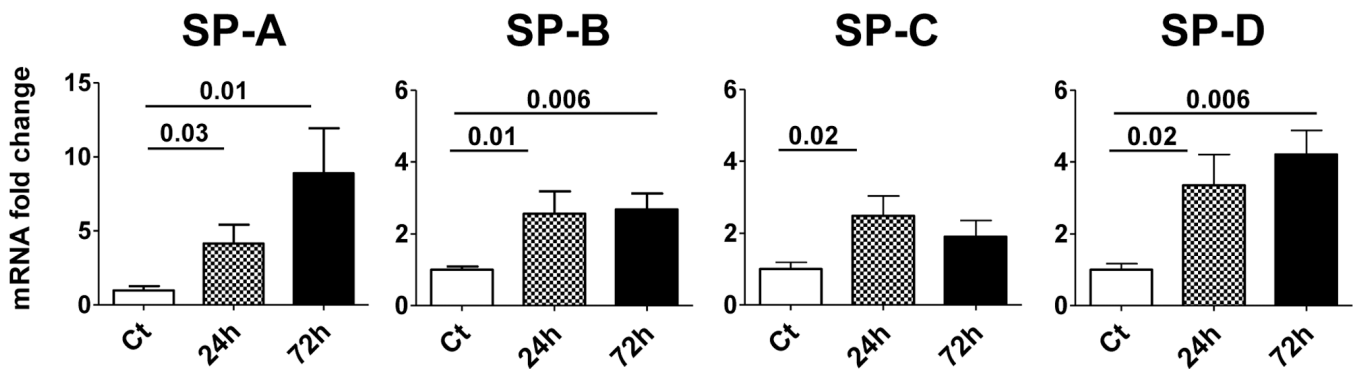

Fig. 3. Intra-amniotic IL-1β induces lung maturation

Surfactant values are expressed as mean (SE) fold change in RNA expression in the lung of each animal (n=4–6/group). Target RNAs were measured by quantitative RT-PCR, and normalized on the expression of eukaryotic 18S rRNA. A sample from the control group was used as a calibrator to calculate the fold changes. Significant *p* values or trends are shown (Mann-Whitney tests).

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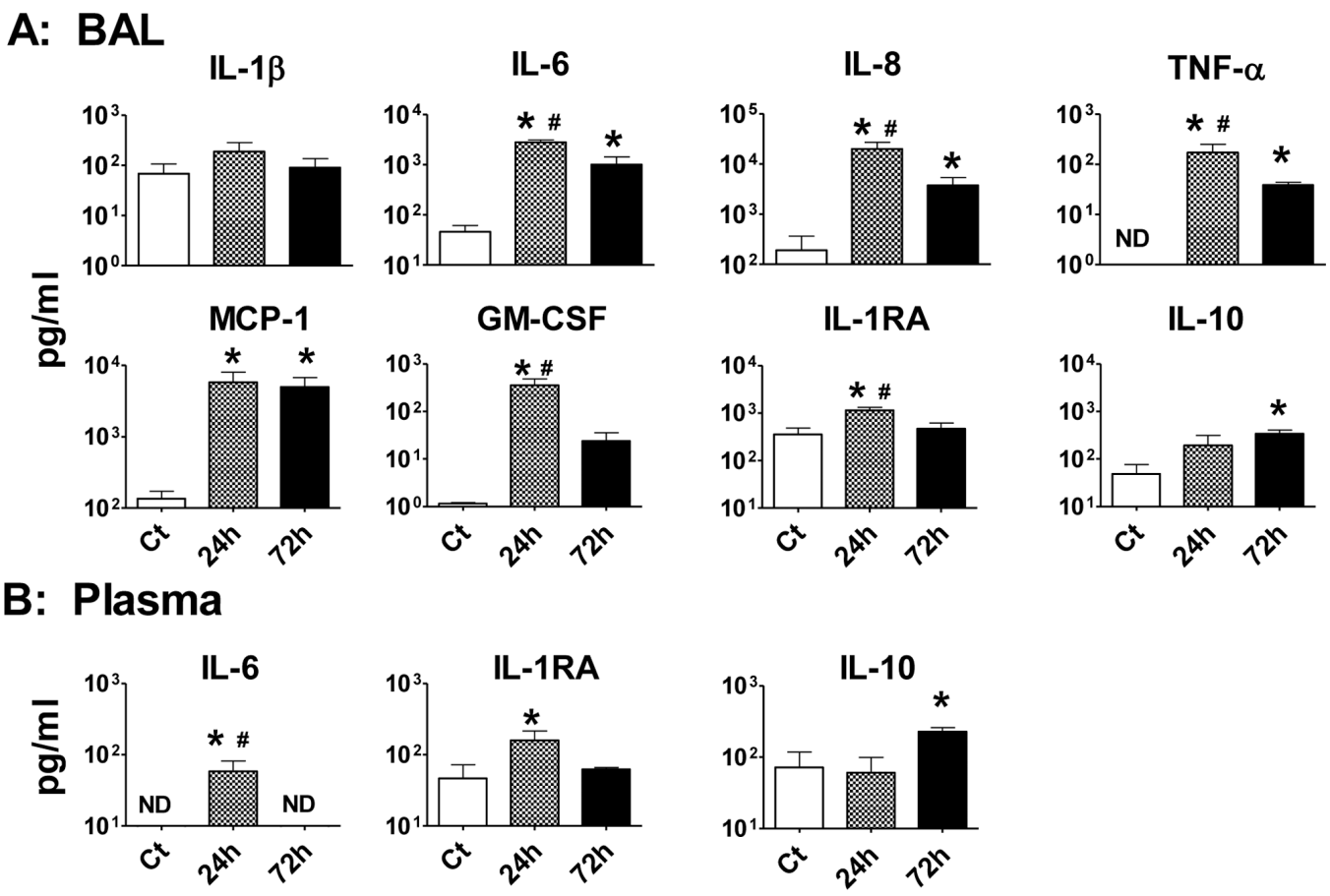

Fig. 4. Median cytokine levels in fetal BAL and plasma

Data are expressed as mean (SE) cytokine levels (pg/ml) in the BAL (A) and plasma (B). For statistical analyses, undetectable values were assigned an arbitrary value of 1 pg/ml. * : Significant difference between controls and IL-1-treated animals (24 h or 72 h, n=4–6/group) (Mann-Whitney tests). $^{\#}$: Significant difference between the 24 h and 72 h time points (Mann-Whitney tests).

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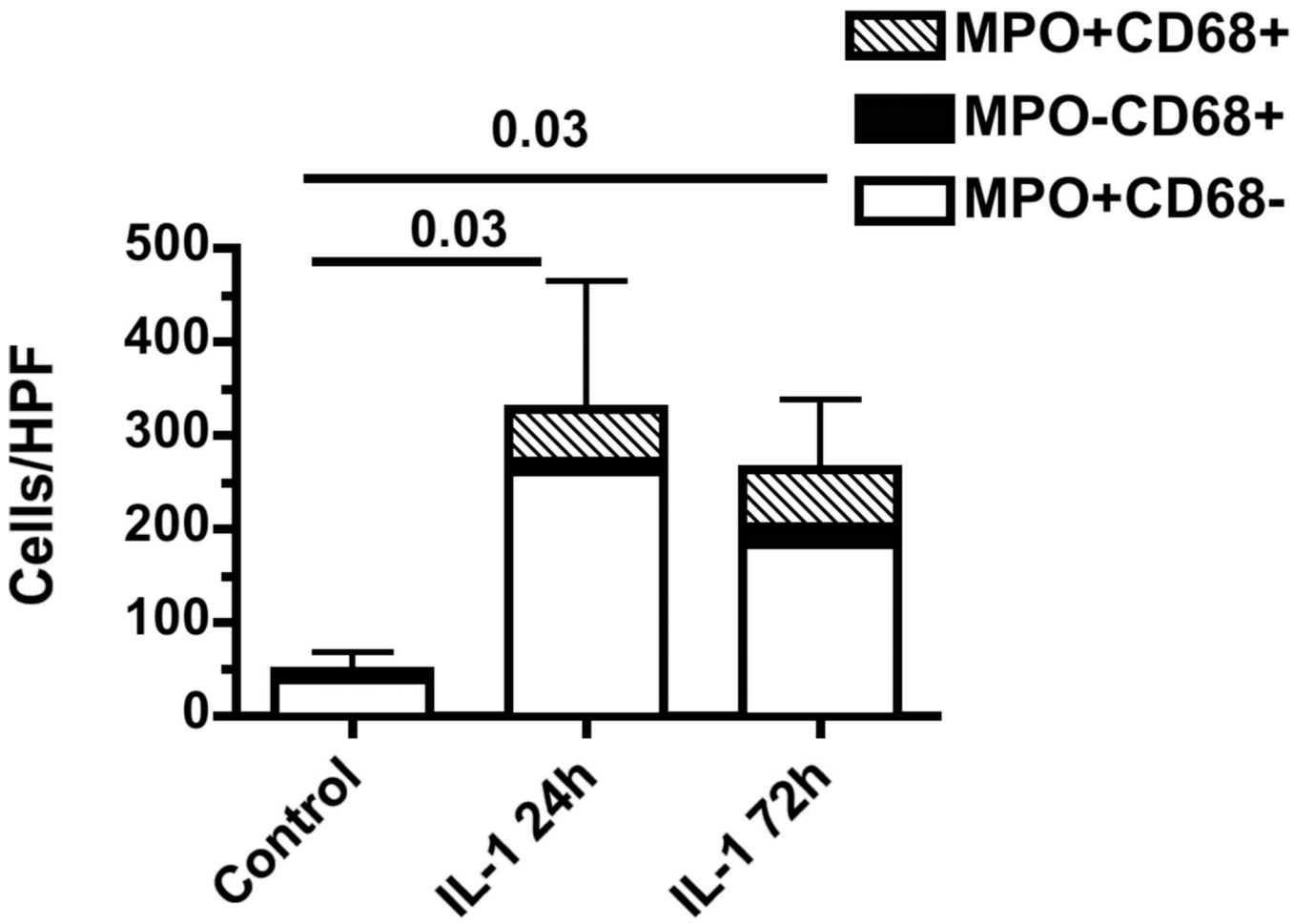

Fig. 5. Intra-amniotic IL-1β induces inflammatory cell influx in the lung

Lung sections were double immunostained with anti-myeloperoxidase (MPO) and anti-CD68. Mean and SE of cell counts per microscopic high power field (CPF) of single and double positive populations are shown. Intra-amniotic IL-1β increased MPO^+ cells and MPO^+CD68^+ cells in the fetal lung (n=4/group). Significant *p* values or trends are shown (Mann-Whitney t-test).

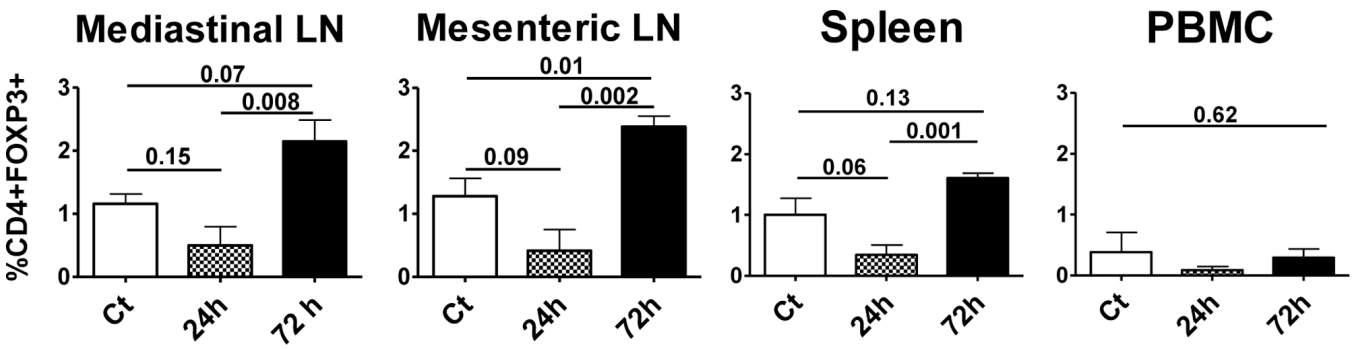

Fig. 6. Intra-amniotic IL-1β transiently decreases Treg cell frequency in lymphatic organs

Values are expressed as mean (SE) Treg cell frequency (defined as percentage of $FOXP3^+$ in the $CD3^+CD4^+$ population) of each organ (n=4–6/group). Significant *p* values or trends are shown (Mann-Whitney tests).

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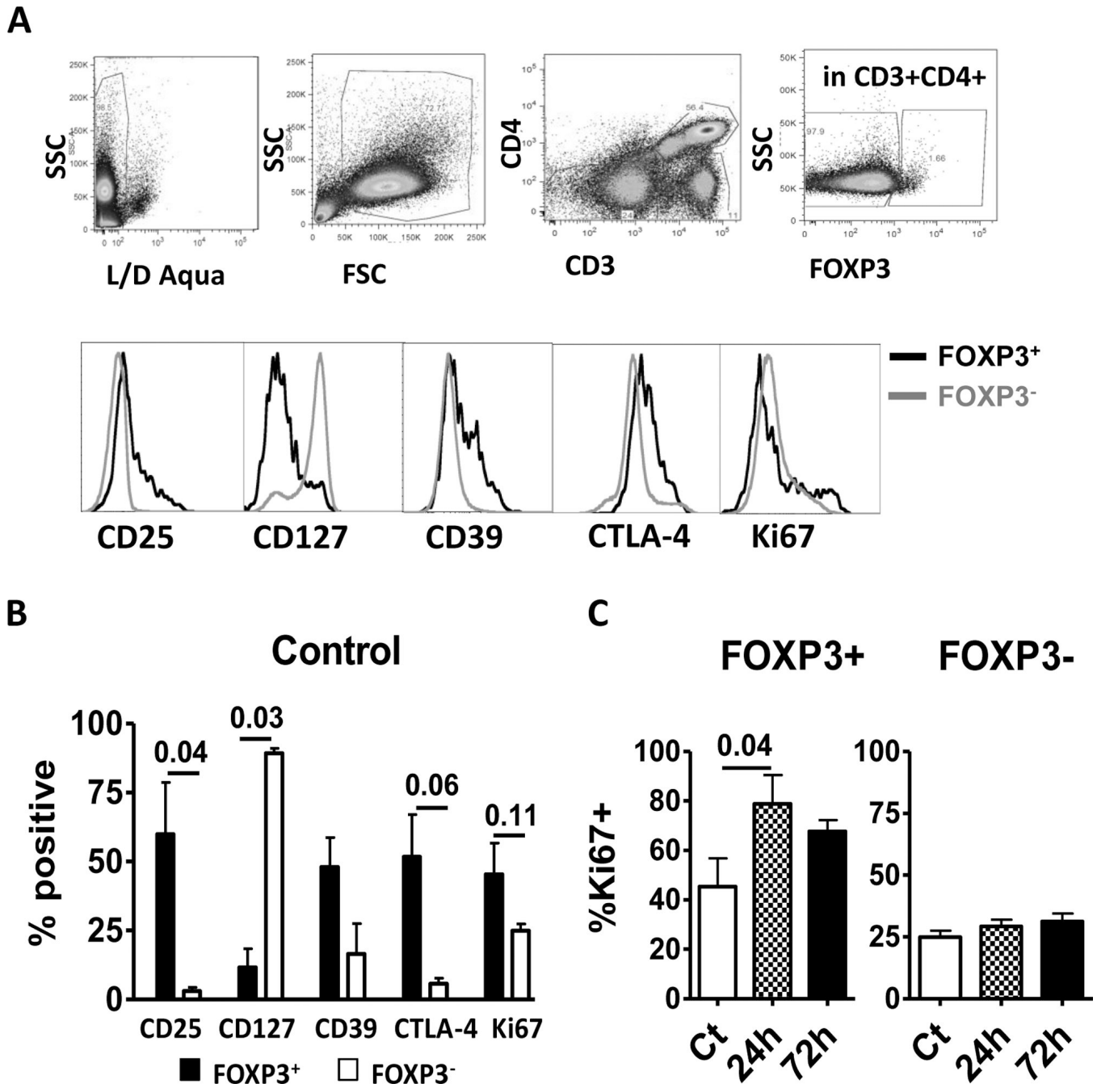

Fig. 7. Phenotypic characterization of Treg and non-Treg cells

(A): Significant example of Treg cell gating. Live cells were first identified by absence of Live/Dead stain, and forward-scatter expression. $CD3^+CD4^+$ T cells were then gated, and $FOXP3^+$ cells were identified in this gate. Cut-off for $FOXP3^+$ cells in $CD3^+CD4^+$ T cells was set up according to a negative biological population, as previously described (35, 36). Expression of CD25, CD127, CD39, CTLA-4 and Ki67 was measured in gated Treg cells ($CD3^+CD4^+FOXP3^+$) or non-Treg cells ($CD3^+CD4^+FOXP3^-$). **(B) Mean (SE) percentage of Treg and non-Treg cells expressing** CD25, CD127, CD39, CTLA-4 and Ki67 in spleens of control animals. Significant *p* values or trends are shown (Mann-Whitney tests). **(C): Effect of IL-1β on Ki67 expression of Treg and non-Treg cells**. Values are expressed as

mean (SE) % of Treg or non-Treg cells expressing Ki67 (n=4–6/group). Significant *p* values or trends are shown (Mann-Whitney tests).

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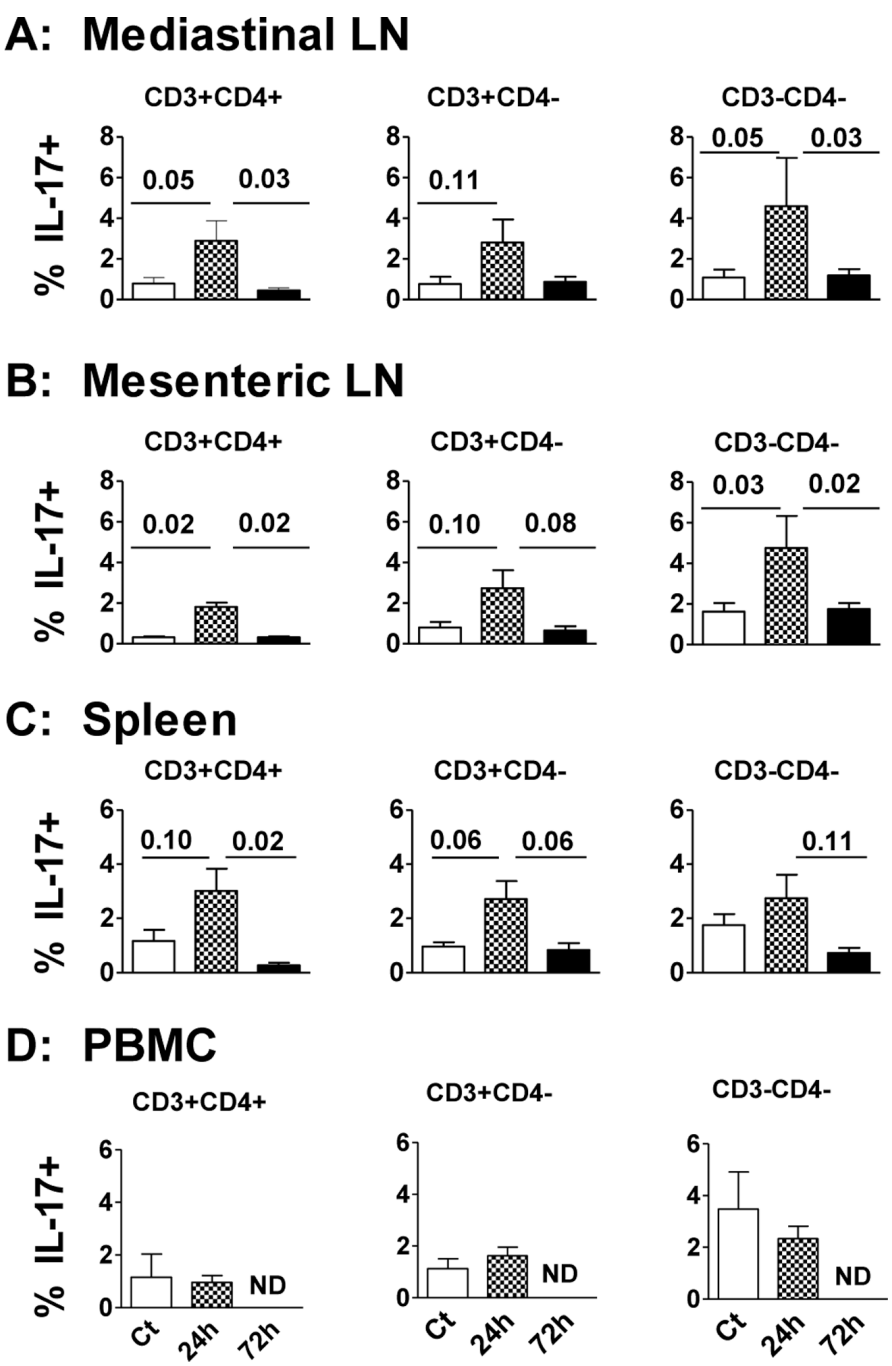

Fig. 8. Intra-amniotic IL-1β transiently increases the frequency of IL-17$^+$ cells in lymphatic organs

Percentages of IL-17A$^+$ cells were measured by flow cytometry in gated CD3$^+$CD4$^+$, CD3$^+$CD4$^-$ and CD3$^-$CD4$^-$ subsets after short *in vitro* polyclonal restimulation of cells purified from the mediastinal LN (A), mesenteric LN (B), spleen (C) and PBMCs (D). In all graphs, values are expressed as mean (SE) % of IL-17$^+$ cells in each subset (n=4–6/group). Significant *p* values or trends are shown (Mann-Whitney tests). ND: not done.

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Table I

Description of the animals included in the study.

	Maternal parity*	Maternal age (yrs)*	Maternal weight (kg)*	Gestational age (days)*	Fetal weight (g)*	Fetus gender (M/F)
Controls (n=7)	4 (3–9)	9 (6–16)	7.4 (5.3–11.3)	131 (121–132)	317 (285–356)	5/2
IL-1 24 h (n=6)	3 (1–12)	8 (7–16)	7.8 (5.4–9.3)	130 (128–132)	324 (263–405)	3/3
IL-1 72 h (n=4)	4 (1–6)	9 (6–11)	8.4 (5.5–10.8)	129 (128–130)	319 (293–443)	1/3
p value †	0.54	0.94	0.89	0.71	0.49	

* Results are expressed as median (range). Maternal weights, ages and parity were recorded at the time the animals were included in the study.

† *p* values correspond to Kruskal-Wallis tests.

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Table II

Median IFN-γ-producing cells in mediastinal LN, mesenteric LN and spleen.

	$CD3^+CD4^+$	$CD3^+CD4^-$	$CD3^-CD4^-$
Mediastinal LN			
Ct	0.7 (0.4–1.9) *	1.3 (0.2–4.0)	1.7 (0.4–2.5)
24h	0.9 (0.2–1.4)	1.1 (0.4–3.4)	3.7 (0.9–7.5)
72h	0.4 (0.2–1.5)	0.5 (0.3–1.3)	2.2 (0.4–3.1)
p #	*0.58*	*0.74*	*0.27*
Mesenteric LN			
Ct	0.6 (0.1–1.4) *	4.24 (0.2–9.9)	3.5 (2.2–7.1)
24h	0.6 (0.4–0.9)	1.41 (0.4–8.4)	1.7 (1.2–3.7)
72h	0.3 (0.1–0.7)	0.53 (0.3–0.8)	4.2 (2.0–5.8)
p	*0.30*	*0.23*	*0.09*
Spleen			
Ct	1.8 (0.5–3.1) *	4.0 (1.0–15.0)	4.6 (4.0–5.8)
24h	1.2 (0.5–6.2)	4.4 (0.8–5.1)	4.1 (1.2–12.4)
72h	0.6 (0.2–2.7)	1.4 (0.7–3.8)	6.1 (4.5–10.8)
p	*0.45*	*0.38*	*0.46*

* Cells purified from the mediastinal LN, mesenteric LN, and spleen of controls (Ct), animals treated by intra-amniotic IL-1β for 24 h (24h) or 72 h (72h) were stimulated *in vitro* with PMA and Ionomycin. Percentages of IFN-γ^+ cells were measured by flow cytometry in gated $CD3^+CD4^+$, $CD3^+CD4^-$ and $CD3^-CD4^-$ subsets. Values are expressed as median (range) % of IFN-γ^+ cells in each subset (n=4–6/group).

p values correspond to Kruskal-Wallis tests.