In Vivo Activation of Invariant Natural Killer T Cells Induces Systemic and Local Alterations in T-Cell Subsets Prior to Preterm Birth

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Running head: Alterations in the T-cell subsets prior to iNKT-cell activation-induced preterm birth

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nuclear factor (NF)-κB, peroxisome proliferator-activated receptor gamma (PPARγ), regulatory T cells (Tregs), subcutaneous (s.c.), T-cell receptor (TCR), T helper cells (Th cells), and uterine-draining lymph nodes (ULNs)

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SUMMARY

Preterm birth, the leading cause of neonatal morbidity and mortality worldwide, is frequently preceded by spontaneous preterm labor, a syndrome of multiple etiologies. Pathological inflammation is causally linked to spontaneous preterm labor. Indeed, direct activation of invariant natural killer T (iNKT) cells via α-galactosylceramide induces preterm labor/birth largely by initiating systemic and local (i.e., decidua and myometrium) innate immune responses. Herein, we investigated whether iNKT-cell activation altered local and systemic T-cell subsets. Administration of α-galactosylceramide induced an expansion of activated CD1d-restricted iNKT cells in the decidua, and a reduction in the number of: 1) total T cells and conventional CD4+ and CD8+ T cells through the down-regulation of the CD3ε molecule in the peripheral circulation, spleen, uterine-draining lymph nodes (ULNs), decidua and/or myometrium; 2) CD4+ regulatory T cells in the spleen, ULNs, and decidua; 3) Th17 cells in the ULNs but an increase in the number of decidual Th17 cells; 4) CD8+ regulatory T cells in the spleen and ULNs, and 5) CD4+ and CD8+ Foxp3- responder T cells in the spleen and ULNs. Since treatment with rosiglitazone prevents iNKT-cell activation-induced preterm labor/birth, we also explored whether the administration of this PPARγ agonist would restore the number of T cells. Treating α-galactosylceramide-injected mice with rosiglitazone partly restored the number of T cells in the spleen but not in the decidua. In summary, iNKT-cell activation altered the systemic and local T–cell

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subsets prior to preterm labor/birth; yet, treatment with rosiglitazone partially reversed such effects.

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INTRODUCTION

Preterm birth — delivery before 37 completed weeks of gestation — is the leading cause of neonatal morbidity and mortality worldwide (1, 2). Two-thirds of all preterm births are preceded by spontaneous preterm labor (3, 4), a syndrome of multiple etiologies (5). Pathological inflammation is implicated in the mechanisms responsible for spontaneous preterm labor (6-8) and is mostly attributed to the activation of the innate limb of immunity (8-16). Indeed, innate lymphocytes, such as invariant natural killer T (iNKT) cells, participate in the inflammatory mechanisms that lead to preterm labor and birth (17-19).

iNKT cells express a CD1d-restricted T cell receptor (TCR) that involves the selective use of Vα14 in mice (20) and Vα24 in humans (21), both of which recognize lipid antigens (22). Activation of iNKT cells can initiate the NF-κB signaling pathway, which leads to a massive immune response mediated by Th1 and Th2 cytokines (23-26). *In vivo* and direct activation of iNKT cells is achieved by the administration of the high-affinity ligand α-galactosylceramide (α-GalCer) (27, 28). Recently, we demonstrated that the *in vivo* and direct activation of iNKT cells via α-GalCer initiates systemic and local (i.e., decidua and myometrium) immune responses leading to preterm labor/birth (19). Such responses are largely mediated by cellular components of the innate immune system, such as neutrophils, macrophages, and dendritic cells (19). Yet, iNKT cells bridge the innate and adaptive limbs of immunity (29); therefore, we propose that such cells have an effect on T cells, the main cellular component of the adaptive immune system. T cells have been implicated in the mechanisms that lead to term (30-34) and

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preterm (31, 32, 35-42) labor. Indeed, *in vivo* activation of T cells through the activation of the CD3 complex induces preterm labor/birth (43). In the current study, we investigated whether iNKT-cell activation via α-GalCer has an effect on systemic and local T-cell subsets prior to preterm labor/birth.

In vivo iNKT-cell activation via α-GalCer downregulates the expression of peroxisome proliferator-activated receptor gamma (PPARγ) target genes such as *Fabp4* and *Fatp4* (19). Yet, treatment with rosiglitazone, a selective PPARγ agonist (44), restores the expression of such genes and reduces the rate of iNKT-cell activation-induced preterm labor/birth (19). Rosiglitazone activates the PPARγ pathway, which interferes with the nuclear factor (NF)-κB, signal transducer and activator of transcription (STAT), and activator protein (AP)-1 signaling pathways, inhibiting the gene transcription of inflammatory mediators (45-47). Herein, we investigated whether treatment with rosiglitazone restores the effect of iNKT-cell activation on local and systemic T-cell subsets.

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MATERIALS AND METHODS

Animals

C57BL/6J (B6) mice were bred in the animal care facility at the C.S. Mott Center for Human Growth and Development (Wayne State University, Detroit, MI, USA) and housed under a circadian cycle (12hrs light:12hrs dark). Females 8-12 weeks old were mated with males of proven fertility. Females were examined daily between 8:00 a.m. and 9:00 a.m., and mating was verified by the presence of a vaginal plug, indicating 0.5 days *post coitum* (dpc). After observation of the vaginal plug, females were separated from the male and placed in new cages. A weight gain of >2 grams by 12.5 dpc confirmed pregnancy. Procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Wayne State University (Protocol Number A-09-08-12).

iNKT-cell activation-induced preterm labor/birth model

Pregnant B6 mice were intravenously (i.v.) injected with 2 μg of α-GalCer (KRN7000; Funakoshi, Tokyo, Japan; n=8) that was dissolved in 50 μL of 4% Dimethyl Sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) or with 50 μL of 4% DMSO alone as a control (n=6) at 16.5 dpc.

Rosiglitazone treatment

Pregnant B6 mice were i.v. injected with 2 μg of α-GalCer at 16.5 dpc (n=8). After 2 h, mice were subcutaneously (s.c.) injected with rosiglitazone (10 mg/kg,

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Selleck Chemicals, Houston, TX, USA) diluted in 1:10 DMSO. Control mice were treated with rosiglitazone alone at 16.5 dpc (n=8).

Tissue collection and leukocyte isolation

Pregnant mice were euthanized 6 h post-α-GalCer injection or post-DMSO, or 4 h post-rosiglitazone treatment (n = 6-8 mice/group). Blood was recovered by cardiac puncture, and the myometrial and decidual tissues were collected and immediately processed for leukocyte isolation. The spleen and uterine-draining lymph nodes (ULNs) were also collected and leukocyte suspensions were prepared.

Leukocyte suspensions from the myometrial and decidual tissues were prepared as follows: tissues were cut into small pieces using fine scissors and enzymatically digested with StemPro Cell Dissociation Reagent (Accutase; Life Technologies, Grand Island, NY, USA) for 35 min at 37°C. Cells from the spleen and ULNs were obtained by gentle dissociation using two glass slides. Leukocyte suspensions from the myometrium, decidua, spleen, and ULNs were then filtered through a 100-μm cell strainer (Fisher Scientific, Hanover Park, IL, USA) and washed with FACS buffer [0.1% bovine serum albumin (Sigma-Aldrich), 0.05% sodium azide (Fisher Scientific, Fair Lawn, NJ, USA), and 1X PBS (Fisher Scientific)].

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Immunophenotyping

Aliquots of 100-150 μL of blood were used for immunophenotyping. Leukocyte suspensions from the myometrium and decidua were stained with the LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Life Technologies) prior to incubation with extracellular mAbs. All of the leukocyte suspensions and blood samples were centrifuged, and cell pellets were incubated for 10 min with CD16/CD32 mAbs (FcγIII/II Receptor, clone 2.4G2; BD Biosciences, San Jose, CA) and subsequently incubated with specific fluorochrome-conjugated anti-mouse mAbs: CD1d-tetramer loaded with α-GalCer-PE (hereafter referred to as CD1d-tetramer, NIH); CD49b/DX5-APC (clone DX5), CD44-APC-Cy7 (clone IM7), CD69-PE-CF594 (clone H1.2F3), CD3ε-APC Cy7 (clone 145-2C11), CD4-APC (clone RM4-5), CD8a-PE-CF594 (clone 53-6.7), CD25-PECy7 (clone PC61), FoxP3-AF488 (clone MF23), and IL17A-AF-700 (clone TC11-18H10) (BD Biosciences) for 30 min. Leukocyte suspensions were fixed/permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA). At least 50,000 events for the splenic, decidual, and blood cells or 25,000 events for the ULNs and myometrial cells were acquired using the BD LSR Fortessa and FACSDiva 8.0 software (both from BD Biosciences). Activated CD1d-restricted iNKT cells and T-cell subsets (cell numbers and mean fluorescence intensity (MFI)) were analyzed within the viability gate. Immunophenotyping included identification of activated CD1d-restricted iNKT cells (CD1d-tetramer+DX5+CD69+CD44+ cells), total T cells (CD3+ cells), conventional CD4+ and CD8+ T cells (CD3+CD4+ and CD3+CD8+ cells), CD4+ regulatory T cells (CD4+ Tregs; CD3+CD4+CD25+Foxp3+ cells),

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CD8+ regulatory T cells (CD8+ Tregs; CD3+CD8+CD25+Foxp3+ cells), CD4+Foxp3- responder T cells (CD3+CD4+CD25+Foxp3- cells), CD8+Foxp3- responder T cells (CD3+CD8+CD25+Foxp3- cells), Th17 cells (CD3+CD4+IL17A+ cells), and double negative T cells (CD3+CD4-CD8- cells). Data were analyzed using the FACSDiva 8.0 software. The total number of T-cell subsets was determined using CountBright Absolute Counting Beads (Molecular Probes, Life Technologies, Eugene, OR, USA). The figures were created using FlowJo software version 10 (TreeStar, Ashland, OR, USA).

Statistical analysis

Flow cytometry data were analyzed using IBM SPSS Version 19.0 (IBM Corporation, Armonk, NY, USA). The statistical significance of group comparisons was assessed using the Mann-Whitney *U* test. A p value of ≤ 0.05 was considered statistically significant.

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RESULTS

Administration of α-GalCer induces iNKT-cell activation in the decidua

First, we confirmed that α-GalCer induced iNKT-cell activation in the decidual tissues (19). Activated CD1d-restricted iNKT cells were identified in the decidua by the expression of the CD1d-tetramer and DX5 antigen as well as the co-expression of CD69 and CD44 antigens (Figure 1A). Administration of α-GalCer induced the proliferation of activated CD1d-restricted iNKT cells in the decidua (Figure 1B).

iNKT-cell activation causes a systemic and local reduction of T cells through the CD3ε molecule prior to preterm labor/birth

We investigated whether iNKT-cell activation via α-GalCer alters systemic and local T cells prior to preterm labor/birth. The gating strategy used to determine total T cells (CD3+ cells) in the tissues and blood is shown in the decidua and ULNs in Supplementary Figure 1. Mice injected with α-GalCer had a reduced number of total T cells in the peripheral blood (Figure 2A A), spleen (Figure 2B), ULNs (Figure 2C), and myometrium (Figure 2D) compared to the DMSO controls. Although not significant, there was a modest reduction in the number of decidual T cells between the mice injected with α-GalCer and the DMSO controls (Figure 2E).

Since we observed a reduction in the total number of T cells, we evaluated the mean fluorescence intensity (MFI) of the CD3ε molecule in leukocytes from the periphery, lymphatic tissues, and the maternal-fetal interface. Administration of α-GalCer down-regulated the expression of the CD3ε molecule in the leukocytes from the periphery(Figure 3A), spleen (Figure 3B), ULNs (Figure 3C), and decidua (Figure 3E).

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Administration of α-GalCer did not have any effect on the CD3ε molecule in the myometrial leukocytes.

In order to investigate whether iNKT-cell activation induced cell death in the decidua, we evaluated the viability of decidual lymphocytes from mice injected with α-GalCer or DMSO. There were no differences in the number of viable cells between these two groups (Supplementary Figure 2).

iNKT-cell activation causes a systemic and local reduction of conventional and regulatory CD4+ T cells prior to preterm labor/birth

Conventional and regulatory CD4+ T cells have been implicated in the processes of term and preterm labor (19, 31, 33, 34, 36-38, 41). We evaluated whether conventional CD4+ T cells would be reduced upon the administration of α-GalCer. The gating strategy used to determine conventional CD4+ T cells in the tissues and blood is shown in Figure 4A. Mice injected with α-GalCer had a lower number of conventional CD4+ T cells in the peripheral blood (Figure 4B), spleen (Figure 4C), ULNs (Figure 4D), and myometrium (Figure 4E) than the DMSO controls. No significant differences were observed in the number of conventional CD4+ T cells between the mice injected with α-GalCer and the DMSO controls in the decidua (Figure 4F).

Next, we evaluated whether there was a systemic and local reduction of CD4+ Tregs prior to iNKT-cell activation-induced preterm labor/birth. The gating strategy used to determine CD4+ Tregs in the spleen, ULNs, and decidua is shown in Figure 5A-C. Mice injected with α-GalCer had a lower number of CD4+ Tregs in the spleen (Figure 5D) and ULNs (Figure 5E) than the DMSO controls. The number of decidual CD4+

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Tregs also decreased in 62.5% (5/8) of the mice injected with α-GalCer; yet, this reduction did not reach statistical significance (Figure 5F). No differences were observed in the number of CD4+ Tregs in the peripheral blood and myometrium upon administration of α-GalCer (data not shown).

iNKT-cell activation alters the number of Th17 cells in the uterine-draining lymph nodes and decidua prior to preterm labor/birth

An imbalance between the effector Th17 cells and CD4+ Tregs may be implicated in the pathogenesis of preterm birth (48). Therefore, we evaluated whether there were systemic and local reductions of Th17 cells prior to iNKT-cell activation-induced preterm labor/birth. The gating strategy used to determine Th17 cells in the ULNs, spleen, and decidua is shown in Figure 6A-C. Mice injected with α-GalCer had a reduced number of Th17 cells in the ULNs (Figure 6D) and spleen (Figure 6E; not significant) compared to the DMSO controls. However, mice injected with α-GalCer had a greater number of decidual Th17 cells than the DMSO controls (Figure 6F). No differences were observed in the number of Th17 cells in the peripheral blood and myometrium upon administration of α-GalCer (data not shown).

iNKT-cell activation causes a systemic and local reduction of CD8+ T cells prior to preterm labor/birth

CD8+ cytotoxic T cells have been implicated in the mechanisms that lead to spontaneous preterm labor (40). Next, we evaluated whether CD8+ T cells were reduced upon administration of α-GalCer. The gating strategy used to determine CD8+

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T cells in the tissues and blood is shown in Figure 7A. Mice injected with α-GalCer had a lower number of CD8+ T cells in the peripheral blood (Figure 7B), spleen (Figure 7C), ULNs (Figure 7D), myometrium (Figure 7E), and decidua (Figure 7F) than the DMSO controls.

iNKT-cell activation causes a reduction of CD8+ regulatory T cells in the spleen and uterine-draining lymph nodes prior to preterm labor/birth

Previous studies have demonstrated that CD8+ Tregs are implicated in the timing of term parturition (34) and endotoxin-induced preterm labor/birth (41). Therefore, we evaluated whether there was a systemic and local reduction of CD8+ Tregs prior to iNKT-cell activation-induced preterm labor/birth. The gating strategy used to determine CD8+ Tregs in the spleen and ULNs is shown in Figure 8A and B. Mice injected with α-GalCer had a lower number of CD8+ Tregs in the spleen (Figure 8C) and ULNs (Figure 8D) than the DMSO controls. No differences were observed in the number of CD8+ Tregs in the peripheral blood, decidua, and myometrium upon administration of α-GalCer (data not shown).

iNKT-cell activation causes alterations in the number of CD4+ and CD8+ Foxp3- responder T cells prior to preterm labor/birth

Since we observed a reduction in the number of CD4+ and CD8+ Tregs, we evaluated whether iNKT-cell activation via α-GalCer altered the number of CD4+ and CD8+ Foxp3- responder T cells. Administration of α-GalCer reduced the number of CD4+ and CD8+ Foxp3- responder T cells in the spleen (Figure 9A and D) and ULNs

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(Figure 9B and E). In the decidua, α-GalCer did not reduce the number of CD4+ Foxp3- responder T cells (Figure 9C); however, it partially decreased the number of CD8+ Foxp3- responder T cells (Figure 9F).

iNKT-cell activation causes a reduction in the number of double negative T cells prior to preterm labor/birth

Lastly, we evaluated whether there were alterations in the number of double negative T cells (CD3+CD4-CD8- cells) prior to iNKT-cell activation-induced preterm labor/birth. The gating strategy used to determine double negative T cells in the tissues and blood is shown in Supplementary Figure 3A. Mice injected with α-GalCer had a reduced number of double negative T cells in the spleen (Supplementary Figure 3C), ULNs (Supplementary Figure 3D), myometrium (Supplementary Figure 3E), and decidua (Supplementary Figure 3F) compared to the DMSO controls. No significant differences were observed in the number of double negative T cells in the peripheral blood between the mice injected with α-GalCer and the DMSO controls (Supplementary Figure 3B).

Treatment with rosiglitazone partially restores the number of T cells in the spleen but not in the decidua

Previous studies demonstrated that treatment with rosiglitazone prevents endotoxin-induced (13) and iNKT-cell activation-induced (19) preterm labor/birth. Herein, we investigated whether treatment with rosiglitazone restores the effect of iNKT-cell activation on systemic and local T cells prior to preterm labor/birth. Treating α-

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GalCer-injected mice with rosiglitazone partially increased the number of total T cells (Figure 10A), CD4+ T cells (Figure 10B), and CD8+ T cells (Figure 10C) in the spleen; however, such increments did not reach statistical significance. In contrast, treatment with rosiglitazone did not restore the T-cell numbers in the decidual tissues (Figure 10D-F). Further, we investigated whether treatment with rosiglitazone restored the number of CD4+ Tregs in the decidua. Treating α-GalCer-injected mice with rosiglitazone did not restore the number of CD4+ Tregs in the decidua (Supplementary Figure 4) and spleen (data not shown).

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DISCUSSION

Principal findings of the study: Administration of α-GalCer induced an expansion of CD1d-restricted iNKT cells in the decidua. Prior to iNKT-cell activation-induced preterm labor/birth, there was 1) a reduction in the number of total T cells, including CD4+ T cells, in the peripheral circulation, spleen, ULNs, and myometrium; 2) a down-regulation in the expression of the CD3ε molecule in the circulating, splenic, lymphatic, and decidual lymphocytes; 3) a decrease in the number of CD4+ Tregs in the spleen, ULNs, and decidua; 4) a reduced number of Th17 cells in the ULNs but an increase in the number of such cells in the decidua; 5) a diminished number of CD8+ T cells in the peripheral circulation, spleen, ULNs, myometrium, and decidua; 6) a reduction in the number of CD8+ Tregs in the spleen and ULNs; and 7) a reduction in the number of CD4+ and CD8+ Foxp3- responder T cells in the spleen and ULNs. Treating α-GalCer-injected mice with rosiglitazone partly restored the number of T cells (CD4+ and CD8+ T cells) in the spleen but not in the decidua. Collectively, these data show that iNKT-cell activation via α-GalCer induces the down-regulation of the CD3ε molecule, which translates to an alteration in the systemic and local T-cell numbers prior to preterm labor/birth; yet, treatment with rosiglitazone partially reversed such effects.

Recently, we demonstrated that *in vivo* T-cell activation by administration of a monoclonal αCD3ε antibody (clone 145-2C11) induces preterm labor/birth (43). This monoclonal αCD3ε antibody activates T cells in the absence of antigens by directly recognizing the CD3ε molecule and evading the T-cell receptor (TCR) antigen-specific interaction (49, 50). *In vitro* and *in vivo* studies have demonstrated that the interaction between αCD3ε and the CD3 molecule initiates endocytosis and a temporary loss of the

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CD3-TCR complex (51-54). Yet, such an interaction simultaneously initiates signaling pathways that result in T-cell activation (55-57). Therefore, *in vivo* and *in vitro* T-cell activation is associated with the temporary loss of the CD3ε molecule (57). In the current study, we found that iNKT-cell activation via α-GalCer caused the down-regulation of the CD3ε molecule, which translated to a reduction in the total number of systemic and local T cells. These findings provide evidence that α-GalCer induces *in vivo* T-cell activation prior to causing preterm labor/birth. In line with this concept, we previously demonstrated that prior to iNKT-cell activation-induced preterm labor/birth, there is an upregulation of the CD25 and PD1 molecules (activation markers) in the myometrial CD4+ T cells (19). In addition, administration of α-GalCer to non-pregnant mice induces the upregulation of CD69 (an early activation marker) in splenocytes (58). Taken together, these results suggest that α-GalCer causes the systemic and local down-regulation of the CD3ε molecule (i.e. T-cell activation) prior to preterm labor/birth.

In the study herein, we found that lymphatic and decidual CD4+ Tregs were reduced prior to iNKT-cell activation-induced preterm labor/birth. CD4+ Tregs are T lymphocytes that express the activation marker CD25 and the transcription factor Foxp3 (59, 60). Their suppressive function is largely due to the expression of Foxp3 (59). During pregnancy, there is an expansion of antigen-specific CD4+ Tregs in the spleen and at the maternal-fetal interface, which promotes maternal-fetal tolerance and pregnancy maintenance (61-64). Indeed, a reduction in the frequency and/or suppressive function of circulating CD4+ Tregs is associated with spontaneous preterm labor (31, 32, 37, 38, 65, 66). In addition, a decline in the number of CD4+ Tregs at the maternal-fetal interface is observed prior to endotoxin-induced preterm labor/birth (41).

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These data support the hypothesis that a breakdown of maternal-fetal tolerance is a mechanism of disease contributing to spontaneous preterm labor (5, 39). Together, these findings allow us to hypothesize that iNKT-cell activation via α-GalCer causes a breakdown of maternal-fetal tolerance by reducing lymphatic and decidual CD4+ Tregs prior to preterm labor/birth. This hypothesis is supported by the fact that activated iNKT cells negatively regulate CD4+ Tregs (67).

Although decidual CD4+Foxp3- responder T cells were not altered prior to iNKT-cell activation-induced preterm labor/birth, the number of decidual Th17 cells was increased. The Th17 cell subset is characterized by the expression of IL17A, IL17F, and IL22 (68). The function of these T cells is wide-ranging since these cells can promote or regulate tissue inflammation (68, 69). Th17 cells are present in the decidual tissues from normal pregnancies (70), and placental hormones (e.g., human chorionic gonadotrophin (71)) may be participating in their proliferation/differentiation. Decidual Th17 cells are also abundant in the chorionic membranes from women who had undergone spontaneous preterm labor with acute histologic chorioamnionitis (72). In fact, an imbalance between effector Th17 cells and CD4+ Tregs has been implicated in the pathogenesis of preterm birth (48). The data presented herein suggest that iNKT-cell activation via α-GalCer induces T-cell activation and therefore promotes the differentiation of effector Th17 cells at the maternal-fetal interface. This observation is concordant with the fact that iNKT-cell activation induces the activation of T cells (19) and iNKT null mice ($J\alpha 281^{-/-}$) have a lower number of Th17 cells (73). Collectively, these findings indicate that, prior to iNKT-cell activation-induced preterm labor/birth, there is an imbalance between CD4+ Tregs and Th17 cells at the maternal-fetal interface.

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In addition to altering the number of Th cell subsets, iNKT-cell activation via α-GalCer reduced the number of lymphatic CD8+ Tregs. Activated CD8+ T cells expressing Foxp3 share phenotypic and functional characteristics with classical CD4+ Tregs (74). These T cells inhibit T-cell responses (e.g., Th17 cells) *in vivo* (75). In late pregnancy, an expansion of peripheral and decidual CD8+ Tregs was observed when IL6 null mice (*Il6*$^{-/-}$) received recombinant IL6 in order to restore the timing of parturition (34). Altogether, these data suggest that CD8+ Tregs can regulate the timing of parturition and that a reduction in the number of these cells may be associated with preterm labor/birth.

Lastly, we showed that treating α-GalCer-injected mice with rosiglitazone, which prevents iNKT-cell activation-induced preterm labor/birth (19), partially restores the T-cell numbers in the spleen. It is well established that PPARγ agonists, such as rosiglitazone, inhibit the activation and proliferation of T cells (76, 77). In our model, we demonstrated that α-GalCer down-regulated PPARγ gene targets; yet, treatment with rosiglitazone restored such effects by activating the PPARγ pathway and preventing preterm labor/birth (19). Therefore, restoration of the T-cell numbers in the spleen may be explained by the fact that rosiglitazone inhibits iNKT-cell proliferation (19), the initial trigger of T-cell activation (i.e., downregulation of the CD3-TCR complex). However, treating α-GalCer-injected mice with rosiglitazone did not restore the number of T cells, including CD4+ Tregs, at the maternal-fetal interface (i.e., decidua). This finding may explain why treatment with rosiglitazone does not prevent preterm birth or improve adverse neonatal outcomes entirely. As an alternative, we are currently investigating whether treatment with a combination of vaginal progesterone and rosiglitazone can

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fully prevent iNKT-cell activation-induced preterm labor/birth. Preliminary results show that this combination may have protective and synergistic effects (Gomez-Lopez et al.; unpublished data). This proposal is based on the finding that treating endotoxin-injected mice with vaginal progesterone increases the frequency of CD4+ Tregs in the decidua and prevents preterm labor/birth (78).

In summary, the study herein showed that iNKT-cell activation via α-GalCer induced T-cell activation (i.e. down-regulation of the CD3ε molecule), which translated to a systemic and local alteration in T-cell subsets prior to preterm labor/birth; yet, treatment with rosiglitazone partially restored such effects (Figure 11). Previously, we demonstrated that *in vivo* iNKT-cell activation induces the activation of the innate immune system (macrophages, dendritic cells and neutrophils) and decidual cells (maternal-fetal interface) prior to preterm labor and birth, which is also attenuated upon treatment with rosiglitazone (19) (Figure 11). Together, these findings provide evidence that both innate and adaptive immune cells are implicated in the pathogenesis of preterm labor and that PPARγ activation can represent a new streategy for the prevention of this syndrome (Figure 11). Yet, a combination of several therapeutic approaches may be required in order to prevent preterm labor/birth entirely and improve adverse neonatal outcomes.

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DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest.

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FIGURE LEGENDS

Figure 1. Administration of α-GalCer induces an expansion of activated CD1d-restricted iNKT cells in decidual tissues. (A) Gating strategy used to identify activated CD1d-restricted iNKT cells (CD1d tetramer+DX5+CD69+CD44+ cells) in decidual tissues. (B) Number of CD1d-restricted iNKT cells in decidual tissues from mice injected with DMSO or α-GalCer (N=6-8 each).

Figure 2. iNKT-cell activation via α-GalCer causes a systemic and local reduction of T cells. The number of T cells in the peripheral blood (A), spleen (B), uterine-draining lymph nodes (ULNs; C), myometrium (D), and decidua (E) from mice injected with DMSO or α-GalCer (N=6-8 each). T cells were gated within the viability gate.

Figure 3. iNKT-cell activation via α-GalCer down-regulates the CD3ε molecule locally and systemically. Mean fluorescence intensity of the CD3ε molecule in leukocytes from the periphery (A), spleen (B), uterine-draining lymph nodes (ULNs; C), myometrium (D), and decidua (E) from mice injected with DMSO or α-GalCer (N=6-8 each).

Figure 4. iNKT-cell activation via α-GalCer causes a systemic and local reduction of CD4+ T cells. The gating strategy used to determine CD4+ T cells (CD3+CD4+CD8- cells) in the peripheral blood and in the lymphatic, myometrial, and decidual tissues (A). CD4+ T cells were gated within the CD3+ and viability gates. The number of CD4+ T cells in the peripheral blood (B), spleen (C), uterine-draining lymph nodes (ULNs; D), myometrium (E), and decidua (F) from mice injected with DMSO or α-GalCer (N=6-8 each).

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Figure 5. iNKT-cell activation via α-GalCer causes a systemic and local reduction of CD4+ Tregs. The gating strategy used to determine CD4+ Tregs (CD3+CD4+CD25+Foxp3+ cells) in the spleen (A), uterine-draining lymph nodes (ULNs; B), and decidua (C). CD4+ Tregs were gated within the CD3+CD4+ and viability gates. The number of CD4+ Tregs in the spleen (D), ULNs (E), and decidua (F) from mice injected with DMSO or α-GalCer (N=6-8 each).

Figure 6. iNKT-cell activation via α-GalCer alters the number of Th17 cells in the uterine-draining lymph nodes and decidua. The gating strategy used to determine Th17 cells (CD3+CD4+IL17A+ cells) in the uterine-draining lymph nodes (ULNs; A), spleen (B), and decidua (C). Th17 cells are defined as CD4+ T cells expression IL17A (right top quadrant). Th17 cells were gated within the CD3+ and viability gates. The number of Th17 cells in the ULNs (D), spleen (E), and decidua (F) from mice injected with DMSO or α-GalCer (N=6-8 each).

Figure 7. iNKT-cell activation via α-GalCer causes the systemic and local reduction of CD8+ T cells. The gating strategy used to determine CD8+ T cells (CD3+CD8+CD4- cells) in the peripheral blood and in the lymphatic, myometrial, and decidual tissues (A). CD8+ T cells were gated within the CD3+ gate. The number of CD8+ T cells in the peripheral blood (B), spleen (C), uterine-draining lymph nodes (ULNs; D), myometrium (E), and decidua (F) from mice injected with DMSO or α-GalCer (N=6-8 each).

Figure 8. iNKT-cell activation via α-GalCer causes a reduction of lymphatic CD8+ Tregs. The gating strategy used to determine CD8+ Tregs (CD3+CD8+CD25+Foxp3+ cells) in the spleen (A) and uterine-draining lymph nodes

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(ULNs; B). CD8+ Tregs were gated within the CD3+CD8+ gate. The number of CD8+ Tregs in the spleen (C) and ULNs (D) from mice injected with DMSO or α-GalCer (N=6-8 each).

Figure 9. iNKT-cell activation via α-GalCer causes a reduction of CD4+ and CD8+ Foxp3- responder T cells in the spleen and uterine-draining lymph nodes. Number of CD4+Foxp3- responder T cells (CD3+CD4+CD25+Foxp3- cells) in the spleen (A), uterine-draining lymph nodes (ULNs; B), and decidua (C) from mice injected with DMSO or α-GalCer (N=6-8 each). Number of CD8+Foxp3- responder T cells (CD3+CD8+CD25+Foxp3- cells) in the spleen (D), uterine-draining lymph nodes (ULNs; E), and decidua (F) from mice injected with DMSO or α-GalCer (N=6-8 each).

Figure 10. Treating α-GalCer-injected mice with rosiglitazone partially restored the T-cell numbers in the spleen but not in the decidua. Number of T cells (A and D), CD4+ T cells (B and E), and CD8+ T cells (C and F) in the spleen and decidua from mice injected with α-GalCer, rosiglitazone (Rosi), or α-GalCer plus rosiglitazone (N=6-8 each).

Figure 11. *In vivo* iNKT-cell activation induces preterm labor/birth by initiating adaptive and innate immune responses. *In vivo* iNKT-cell activation via α-GalCer induces T-cell activation (down-regulation of the CD3ε molecule), which translates into alterations in T-cell subsets at the maternal-fetal interface prior to preterm labor and birth. In addition, *in vivo* iNKT-cell activation induces the activation of the innate immune system and decidual cells (maternal-fetal interface) prior to preterm labor and birth (19). PPARγ activation via treatment with rosiglitazone could partially restore such effects and prevent preterm labor and birth.

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Figure 10

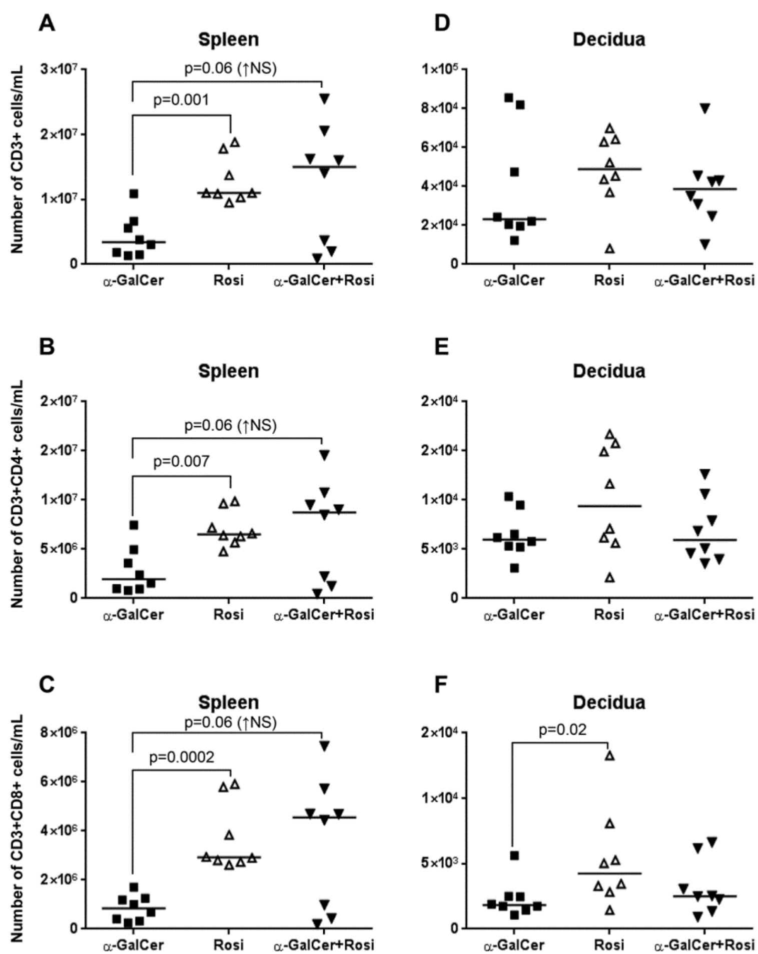

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Figure 9

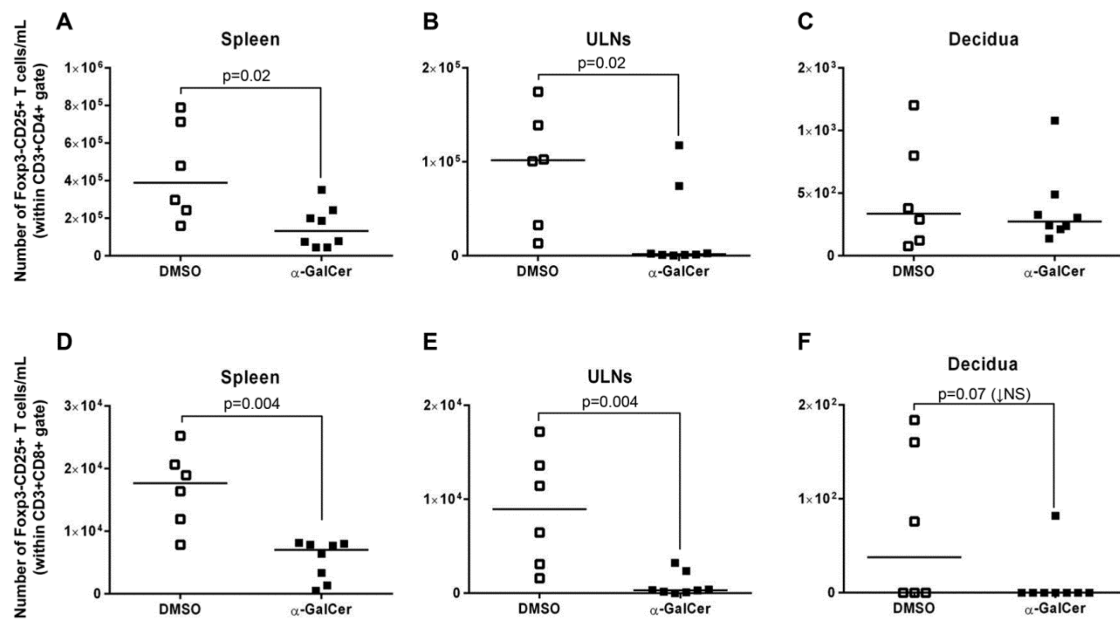

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Figure 8

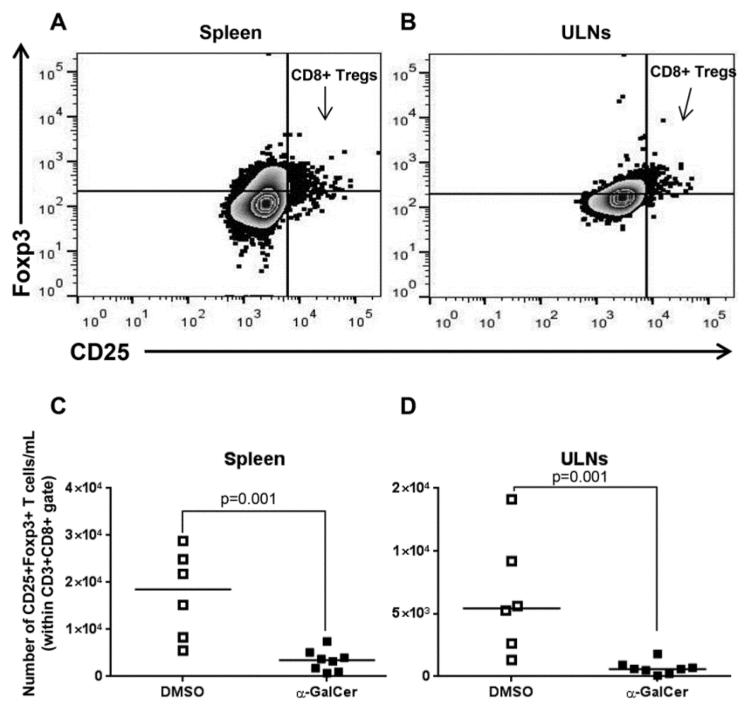

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Figure 7

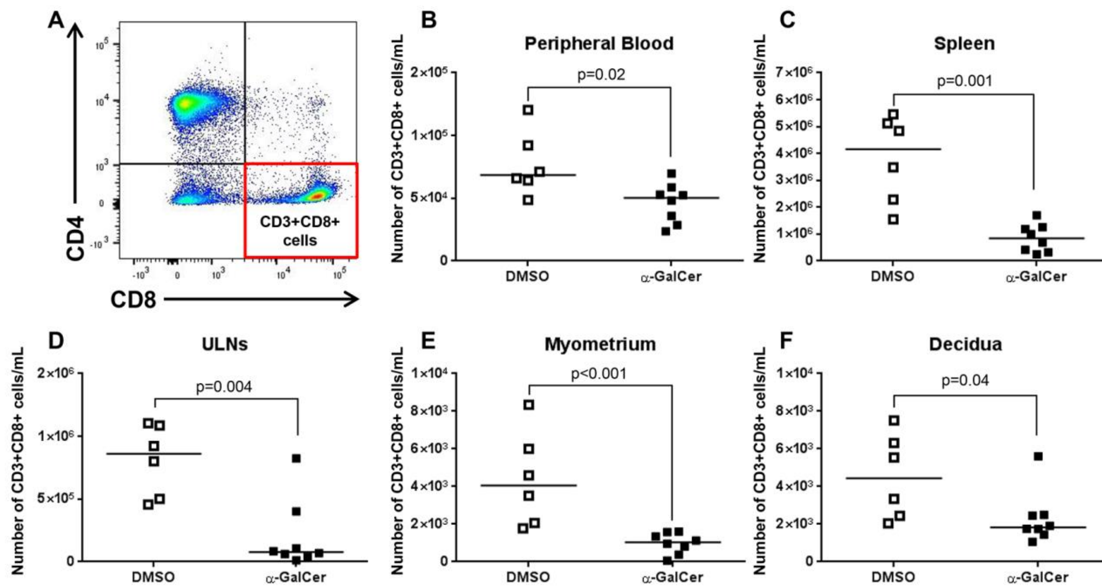

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Figure 6

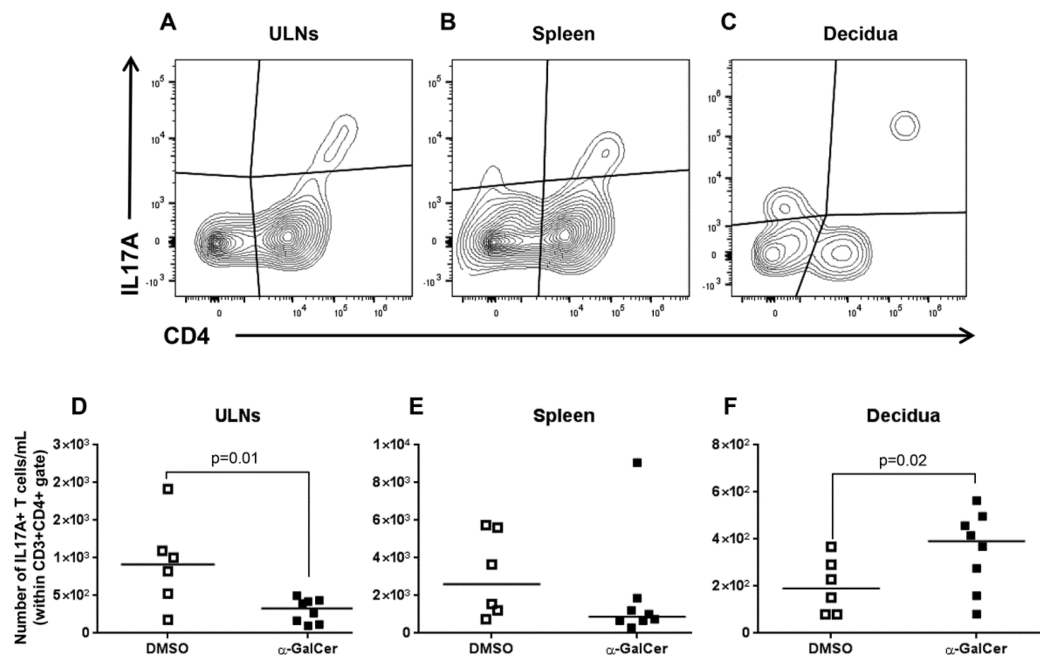

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Figure 5

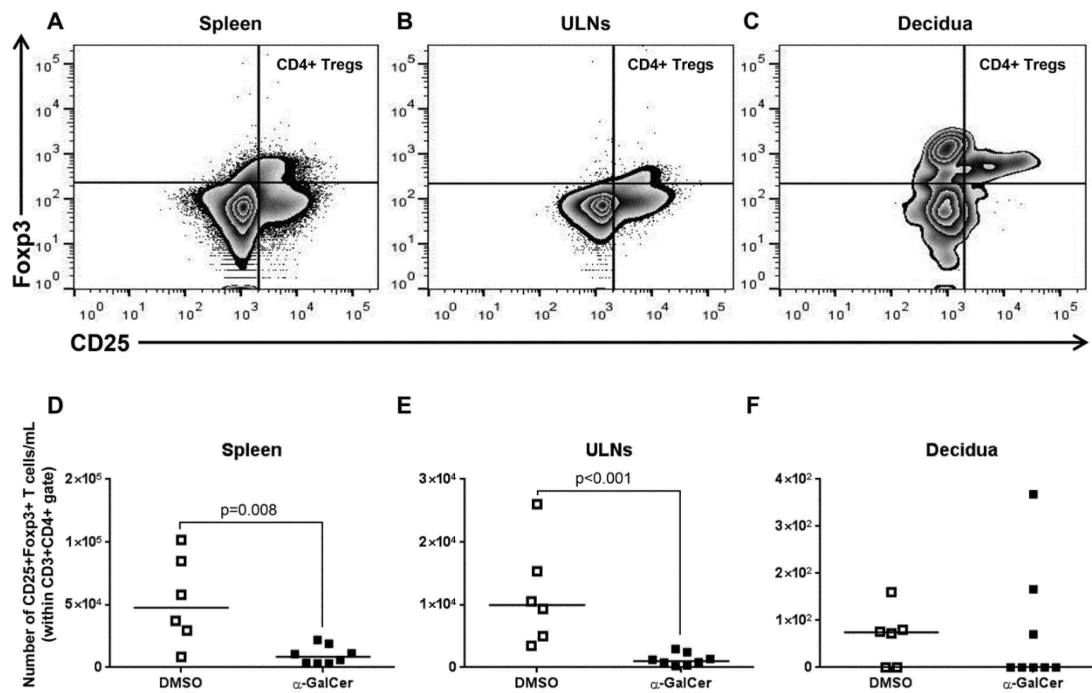

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Figure 4

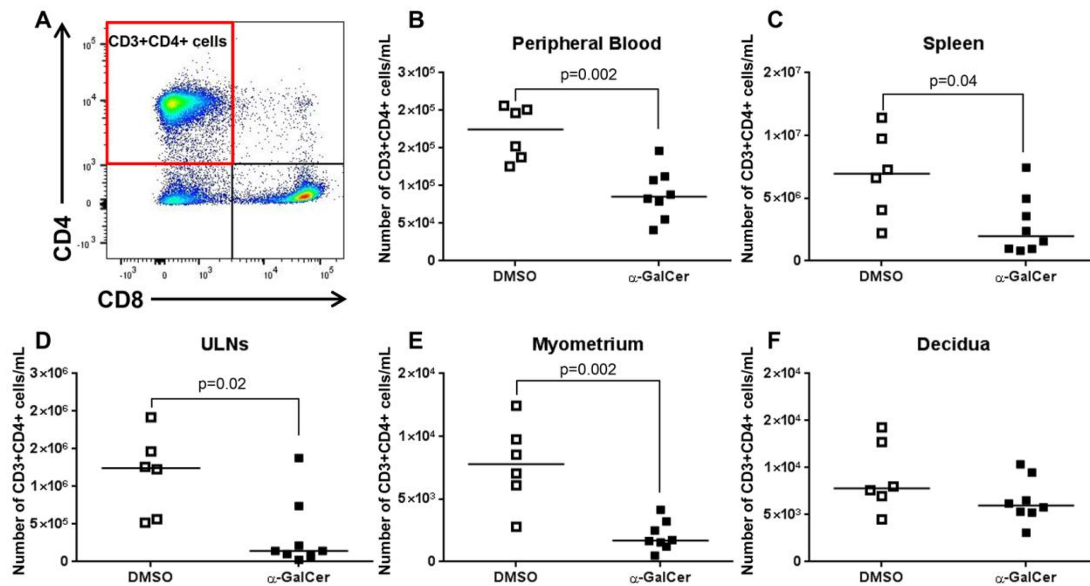

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Figure 3

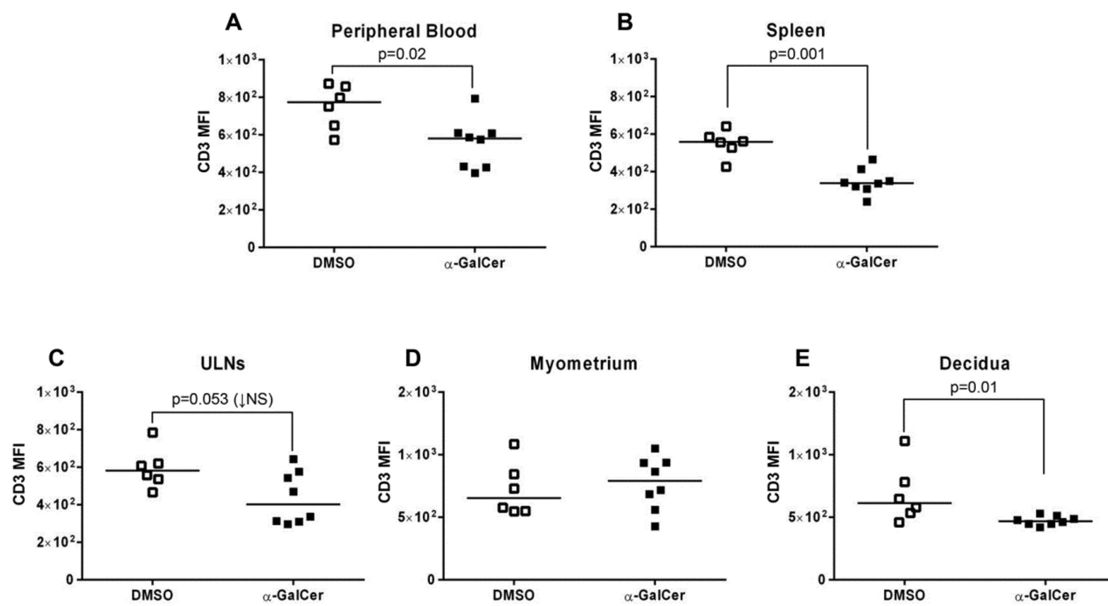

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Figure 2

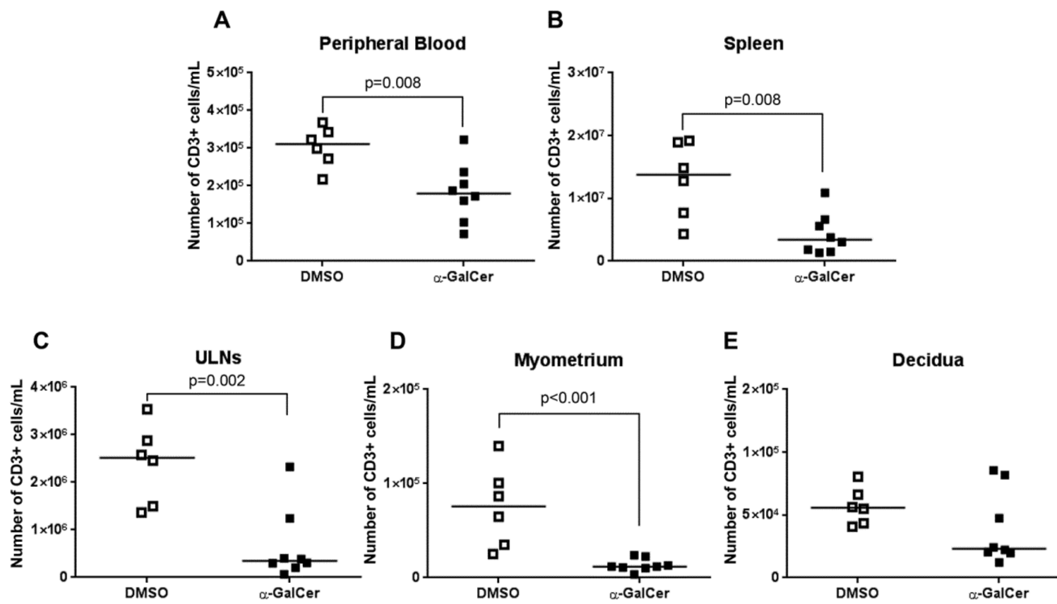

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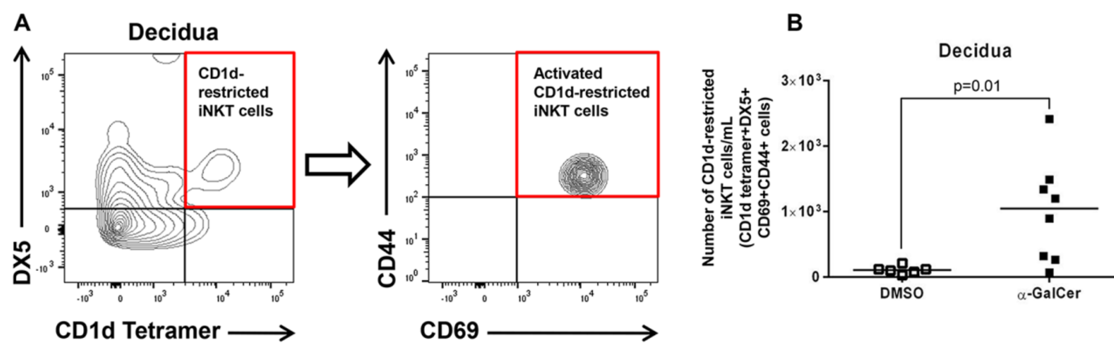
Figure 1



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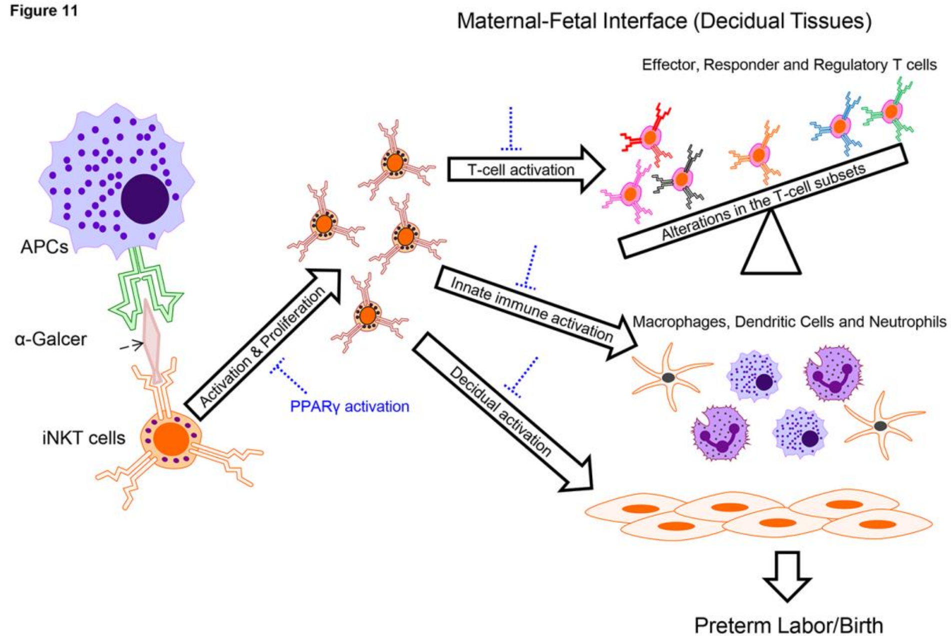

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