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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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Summary

Preterm birth, the leading cause of neonatal morbidity and mortality worldwide, is frequently preceded by spontaneous preterm labour, a syndrome of multiple aetiologies. Pathological inflammation is causally linked to spontaneous preterm labour. Indeed, direct activation of invariant natural killer T (iNKT) cells via α-galactosylceramide induces preterm labour/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 (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) T helper type 17 (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 $CD8^+$ forkhead box protein 3 negative ($Foxp3^-$) responder T cells in the spleen and ULNs. As treatment with rosiglitazone prevents iNKT-cell activation-induced preterm labour/birth, we also explored whether the administration of this peroxisome proliferator-activated receptor gamma (PPARγ) agonist would restore the number of T cells. Treating α-galactosylceramide-injected mice with rosiglitazone partially 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 subsets prior to preterm labour/birth; however, treatment with rosiglitazone partially reversed such effects.

Keywords: cytokine, inflammation, parturition, pregnancy, prematurity, preterm labour, PPARγ, rosiglitazone

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 labour [3,4], a syndrome of multiple aetiologies [5]. Pathological inflammation is implicated in the mechanisms responsible for spontaneous preterm labour [6–8] and is mainly 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 labour 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 nuclear factor kappa B (NF-κB) signalling pathway, which leads to a massive immune response mediated by T helper type 1 (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 labour/birth [19]. Such responses are largely mediated by cellular components of the innate immune system, such as neutrophils, macrophages and dendritic cells [19]. However, 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 preterm [31,32,35–42] labour. Indeed, *in vivo* activation of T cells through the stimulation of the CD3 complex induces preterm labour/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 labour/birth.

In vivo iNKT-cell activation via α-GalCer down-regulates the expression of peroxisome proliferator-activated receptor gamma (PPARγ) target genes such as *Fabp4* and *Fatp4* [19]. However, treatment with rosiglitazone, a selective PPARγ agonist [44], restores the expression of such genes and reduces the rate of iNKT-cell activation-induced preterm labour/birth [19]. Rosiglitazone activates the PPARγ pathway, which interferes with the NF-κB, signal transducer and activator of transcription (STAT), and activator protein (AP)-1 signalling 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.

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 (12-h light/12-h 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 males and placed into new cages. A weight gain of > 2 g 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 labour/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 sulphoxide (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; 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 leucocyte isolation

Pregnant mice were euthanized 6 h post-α-GalCer or post-DMSO injection, 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 leucocyte isolation. The spleen and uterine-draining lymph nodes (ULNs) were also collected and leucocyte suspensions were prepared.

Leucocyte 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. Leucocyte 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 fluorescence activated cell sorter (FACS) buffer [0·1% bovine serum albumin (Sigma-Aldrich), 0·05% sodium azide (Fisher Scientific, Fair Lawn, NJ, USA) and 1× phosphate-buffered saline (PBS; Fisher Scientific)].

Immunophenotyping

Aliquots of 100–150 μL of blood were used for immunophenotyping. Leucocyte 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 monoclonal antibodies (mAbs). All leucocyte 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, USA) and subsequently incubated with specific fluorochrome-conjugated anti-mouse mAbs: CD1d-tetramer loaded with α-GalCer-phycoerythrin (PE) (hereafter referred to as CD1d-tetramer, NIH), CD49b/DX5-allophycocyanin (APC) (clone DX5), CD44-APC-cyanin 7 (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-PE-Cy7 (clone PC61), forkhead box protein 3 (Foxp3)-AF488

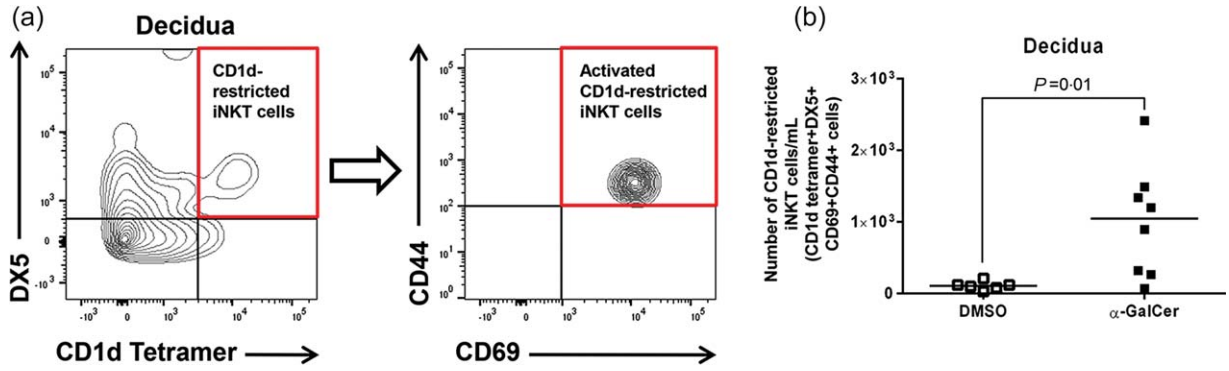

Fig. 1. Administration of α-galactosylceramide (α-GalCer) induces an expansion of activated CD1d-restricted invariant natural killer (iNKT) cells in decidual tissues. (a) Gating strategy used to identify activated CD1d-restricted iNKT cells (CD1d tetramer$^+$DX5$^+$CD69$^+$CD44$^+$ cells) in decidual tissues. (b) Number of CD1d-restricted iNKT cells in decidual tissues from mice injected with dimethyl sulphoxide (DMSO, control) or α-GalCer ($n = 6$–8 each). [Colour figure can be viewed at wileyonlinelibrary.com].

(clone MF23) and interleukin (IL)-17A-AF-700 (clone TC11-18H10) (BD Biosciences) for 30 min. Leucocyte 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 version 8.0 software (both from BD Biosciences). Activated CD1d-restricted iNKT cells and T-cell subsets [cell numbers and mean fluorescence intensity (MFI)] were analysed 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$^+$ T_{regs}; CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$ cells), CD8$^+$ regulatory T cells (CD8$^+$$T_{regs}$; CD3$^+$CD8$^+$CD25$^+$Foxp3$^+$ 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$^+$IL-17A$^+$ cells) and double-negative T cells (CD3$^+$CD4$^-$CD8$^-$ cells). Data were analysed using the FACSDiva version 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 analysed using the IBM SPSS software version 19 (IBM Corporation, Armonk, NY, USA). The statistical significance of group comparisons was assessed using Mann–Whitney *U*-tests. A *P*-value of $\leq 0{\cdot}05$ was considered statistically significant.

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 (Fig. 1a). Administration of α-GalCer induced the proliferation of activated CD1d-restricted iNKT cells in the decidua (Fig. 1b).

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

We investigated whether iNKT-cell activation via α-GalCer alters systemic and local T cells prior to preterm labour/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 Supporting information, Fig. S1. Mice injected with α-GalCer had a reduced number of total T cells in the peripheral blood (Fig. 2a), spleen (Fig. 2b), ULNs (Fig. 2c) and myometrium (Fig. 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 (Fig. 2e).

As we observed a reduction in the total number of T cells, we evaluated the mean fluorescence intensity (MFI) of the CD3ε molecule in leucocytes from the periphery, lymphatic tissues and maternal–fetal interface. Administration of α-GalCer down-regulated the expression of the CD3ε molecule in the leucocytes from the periphery (Fig. 3a), spleen (Fig. 3b), ULNs (Fig. 3c) and decidua (Fig. 3e).

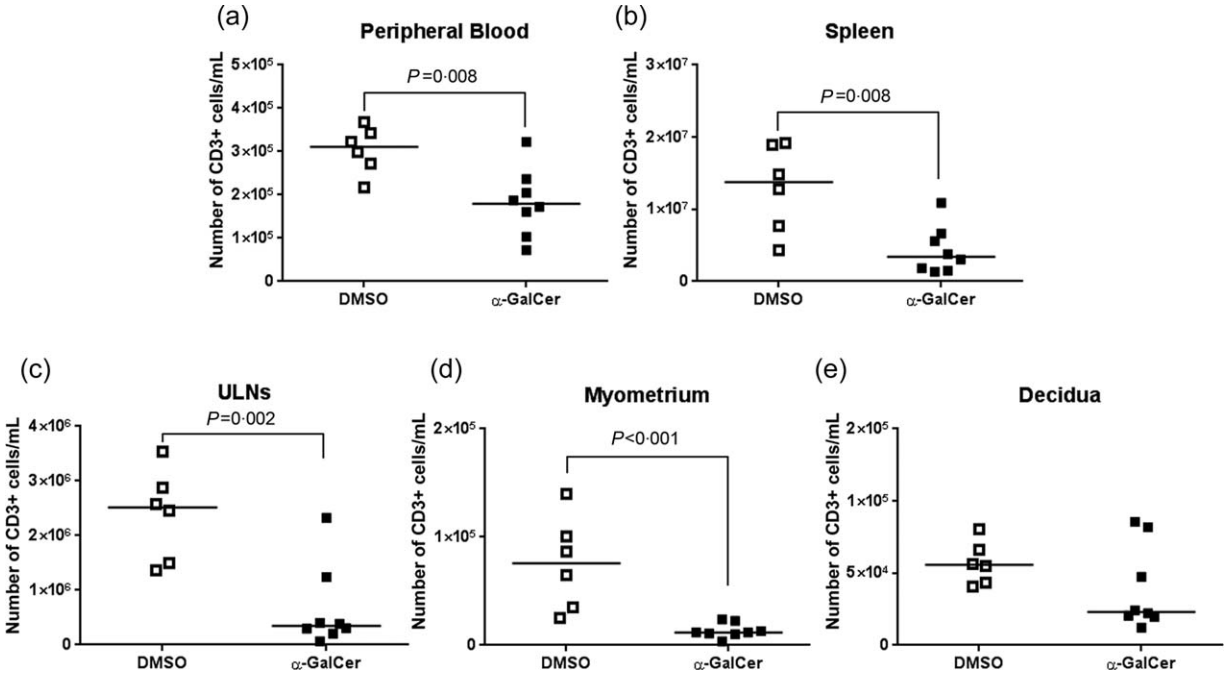

Fig. 2. Invariant natural killer (iNKT)-cell activation via α-galactosylceramide (α-GalCer) causes a systemic and local reduction of T cells. The number of T cells in the peripheral blood (a), spleen (b), uterine-draining lymph nodes (ULNs; c), myometrium (d) and decidua (e) from mice injected with dimethyl sulphoxide (DMSO, control) or α-GalCer ($n = 6$–8 each). T cells were gated within the viability gate.

Administration of α-GalCer did not have any effect on the CD3ε molecule in the myometrial leucocytes (Fig. 3d).

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 (Supporting information, Fig. S2).

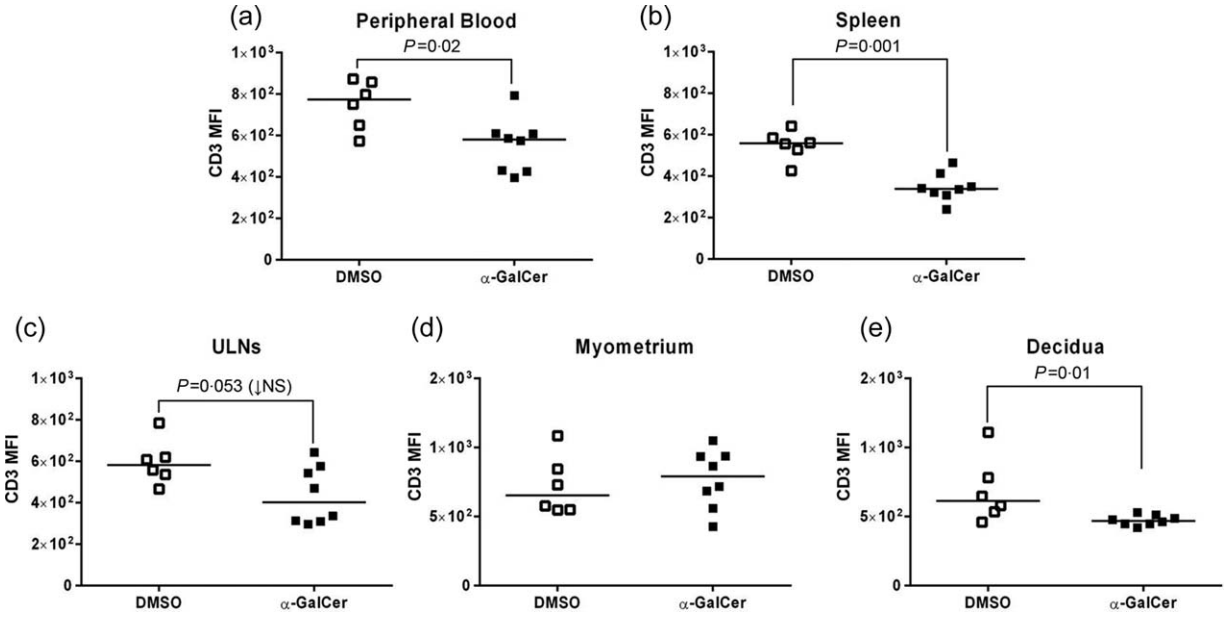

Fig. 3. Invariant natural killer (iNKT)-cell activation via α-galactosylceramide (α-GalCer) down-regulates the CD3ε molecule locally and systemically. Mean fluorescence intensity (MFI) of the CD3ε molecule in leucocytes from the periphery (a), spleen (b), uterine-draining lymph nodes (ULNs; c), myometrium (d), and decidua (e) from mice injected with dimethyl sulphoxide (DMSO, control) or α-GalCer ($n = 6$–8 each).

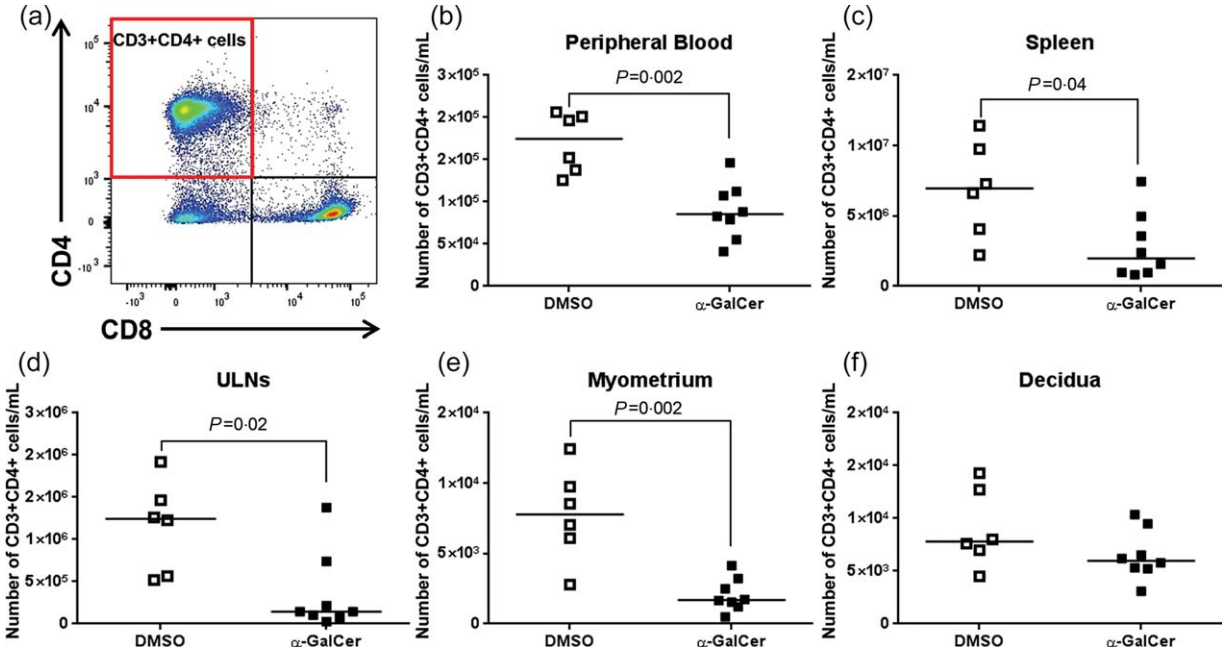

Fig. 4. Invariant natural killer (iNKT)-cell activation via α-galactosylceramide (α-GalCer) causes a systemic and local reduction of $CD4^+$ T cells. The gating strategy used to determine $CD4^+$ T cells ($CD3^+CD4^+CD8^-$ cells) in the peripheral blood and in the lymphatic, myometrial and decidual tissues (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 dimethyl sulphoxide (DMSO, control) or α-GalCer ($n = 6$–8 each). [Colour figure can be viewed at wileyonlinelibrary.com].

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

Conventional and regulatory $CD4^+$ T cells have been implicated in the processes of term and preterm labour [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 Fig. 4a. Mice injected with α-GalCer had a lower number of conventional $CD4^+$ T cells in the peripheral blood (Fig. 4b), spleen (Fig. 4c), ULNs (Fig. 4d) and myometrium (Fig. 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 (Fig. 4f).

Next, we evaluated whether there was a systemic and local reduction of $CD4^+$ T_{regs} prior to iNKT-cell activation-induced preterm labour/birth. The gating strategy used to determine $CD4^+$ T_{regs} in the spleen, ULNs and decidua is shown in Fig. 5a–c. Mice injected with α-GalCer had a lower number of $CD4^+$ T_{regs} in the spleen (Fig. 5d) and ULNs (Fig. 5e) than the DMSO controls. The number of decidual $CD4^+$ T_{regs} also decreased in 62·5% (five out of eight) of the mice injected with α-GalCer; however, this reduction did not reach statistical significance (Fig. 5f). No differences were observed in the number of $CD4^+$ T_{regs} 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 labour/birth

An imbalance between the effector Th17 cells and $CD4^+$ T_{regs} 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 labour/birth. The gating strategy used to determine Th17 cells in the ULNs, spleen and decidua is shown in Fig. 6a–c. Mice injected with α-GalCer had a reduced number of Th17 cells in the ULNs (Fig. 6d) and spleen (Fig. 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 (Fig. 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 labour/birth

$CD8^+$ cytotoxic T cells have been implicated in the mechanisms that lead to spontaneous preterm labour [40]. Next,

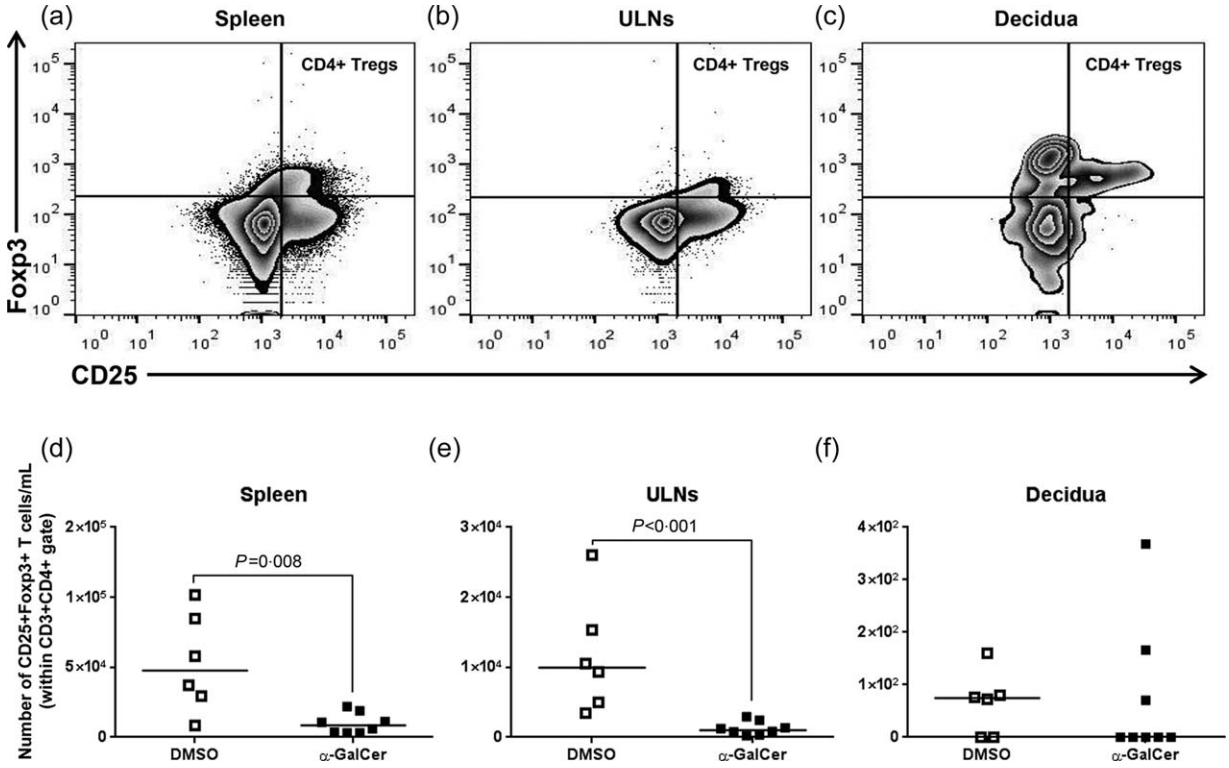

Fig. 5. Invariant natural killer (iNKT)-cell activation via α-galactosylceramide (α-GalCer) causes a systemic and local reduction of $CD4^+$ regulatory T cells (T_{regs}). The gating strategy used to determine $CD4^+$ T_{regs} ($CD3^+CD4^+CD25^+$ forkhead box protein 3 ($Foxp3^+$) cells) in the spleen (a), uterine-draining lymph nodes (ULNs; b) and decidua (c). $CD4^+$ T_{regs} were gated within the $CD3^+CD4^+$ and viability gates. The number of $CD4^+$ T_{regs} in the spleen (d), ULNs (e) and decidua (f) from mice injected with dimethyl sulphoxide (DMSO, control) or α-GalCer ($n = 6$–8 each).

we evaluated whether $CD8^+$ T cells were reduced upon administration of α-GalCer. The gating strategy used to determine $CD8^+$ T cells in the tissues and blood is shown in Fig. 7a. Mice injected with α-GalCer had a lower number of $CD8^+$ T cells in the peripheral blood (Fig. 7b), spleen (Fig. 7c), ULNs (Fig. 7d), myometrium (Fig. 7e) and decidua (Fig. 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 labour/birth

Previous studies have demonstrated that $CD8^+$ T_{regs} are implicated in the timing of term parturition [34] and endotoxin-induced preterm labour/birth [41]. Therefore, we evaluated whether there was a systemic and local reduction of $CD8^+$ T_{regs} prior to iNKT-cell activation-induced preterm labour/birth. The gating strategy used to determine $CD8^+$ T_{regs} in the spleen and ULNs is shown in Fig. 8a,b. Mice injected with α-GalCer had a lower number of $CD8^+$ T_{regs} in the spleen (Fig. 8c) and ULNs (Fig. 8d) than the DMSO controls. No differences were observed in the number of $CD8^+$ T_{regs} 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 labour/birth

As we observed a reduction in the number of $CD4^+$ and $CD8^+$ T_{regs}, 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 (Fig. 9a,d) and ULNs (Fig. 9b,e). In the decidua, α-GalCer did not reduce the number of $CD4^+Foxp3^-$ responder T cells (Fig. 9c); however, it partially decreased the number of $CD8^+Foxp3^-$ responder T cells (Fig. 9f).

iNKT-cell activation causes a reduction in the number of double-negative T cells prior to preterm labour/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 labour/birth. The gating strategy used to determine double-negative T cells in the tissues and blood is shown in Supporting information, Fig. S3a. Mice injected with α-GalCer had a reduced number of double-negative T cells in the

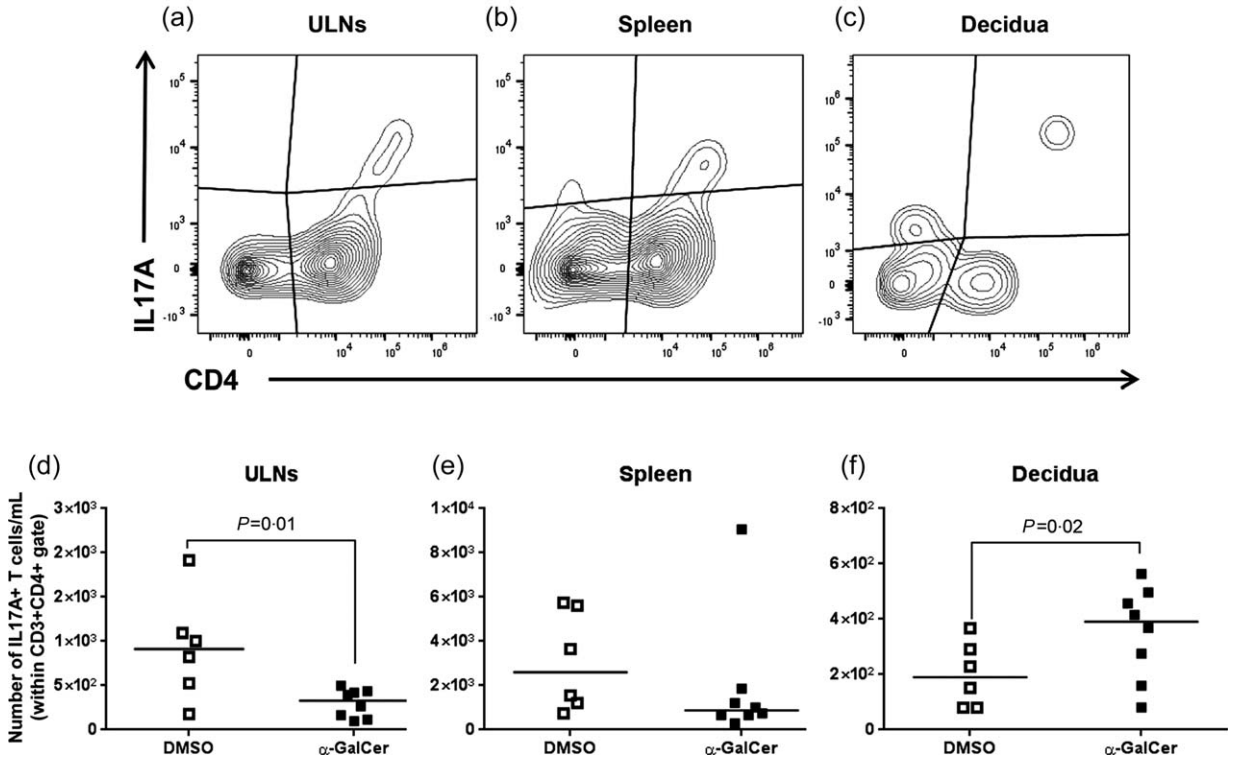

Fig. 6. Invariant natural killer (iNKT)-cell activation via α-galactosylceramide (α-GalCer) alters the number of T helper type 17 (Th17) cells in the uterine-draining lymph nodes (ULNs) and decidua. The gating strategy used to determine Th17 cells [$CD3^+CD4^+$interleukin (IL)-$17A^+$ cells] in the ULNs (a), spleen (b) and decidua (c). Th17 cells are defined as $CD4^+$ T cells that express IL-17A (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 dimethyl sulphoxide (DMSO, control) or α-GalCer ($n = 6$–8 each).

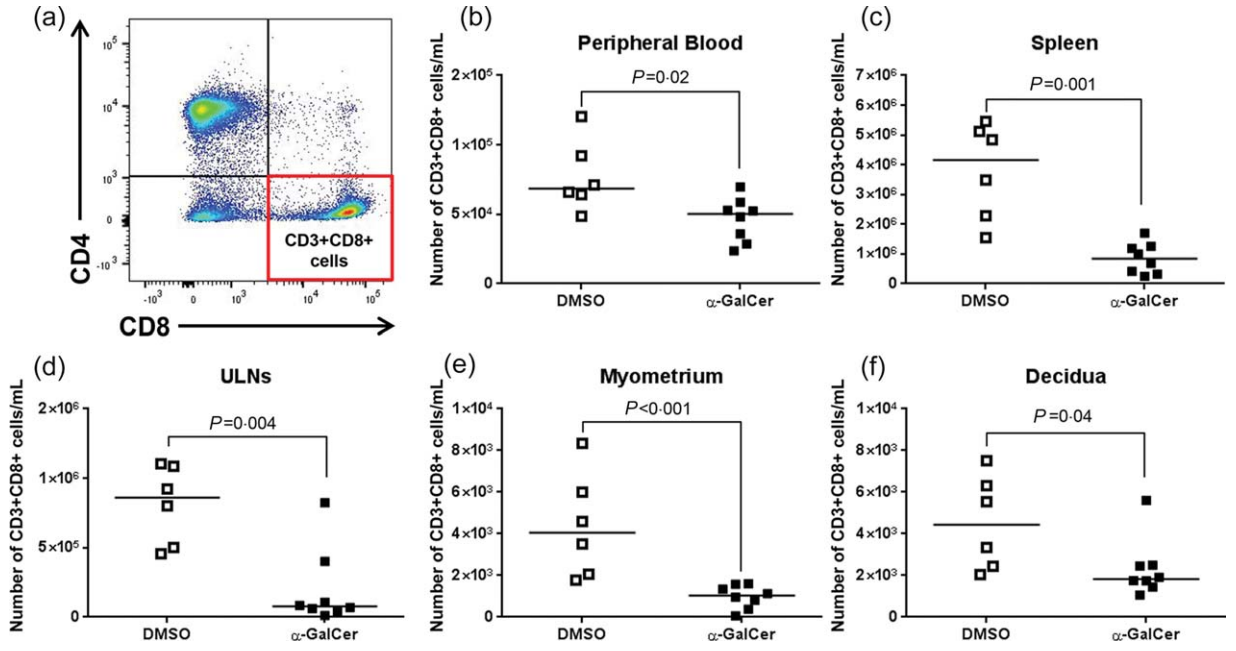

Fig. 7. Invariant natural killer (iNKT)-cell activation via α-galactosylceramide (α-GalCer) causes the systemic and local reduction of $CD8^+$ T cells. The gating strategy used to determine $CD8^+$ T cells ($CD3^+CD8^+CD4^-$ cells) in the peripheral blood and in the lymphatic, myometrial, and decidual 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 dimethyl sulphoxide (DMSO, control) or α-GalCer ($n = 6$–8 each). [Colour figure can be viewed at wileyonlinelibrary.com].

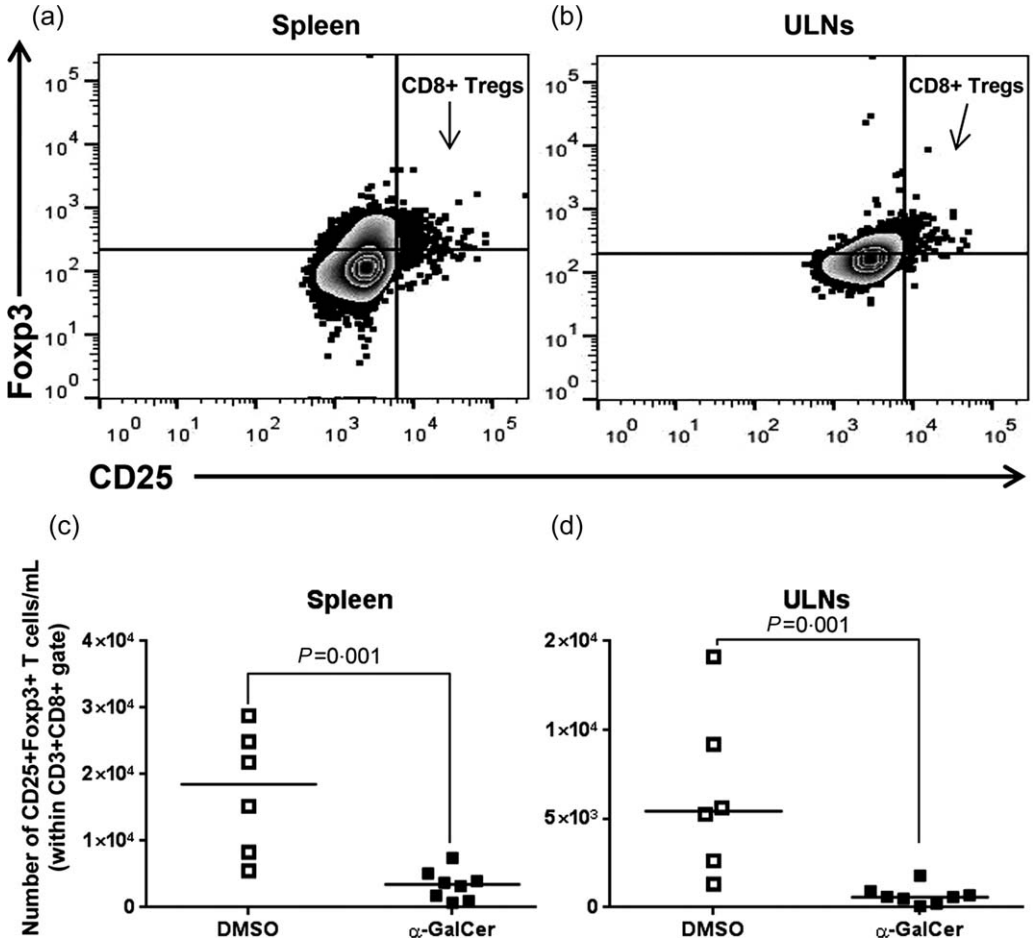

Fig. 8. Invariant natural killer (iNKT)-cell activation via α-galactosylceramide (α-GalCer) causes a reduction of lymphatic $CD8^+$ regulatory T cells (T_{regs}). The gating strategy used to determine $CD8^+$ T_{regs} [$CD3^+CD8^+CD25^+$ forkhead box protein 3 ($Foxp3^+$) cells] in the spleen (a) and uterine-draining lymph nodes (ULNs; b). $CD8^+$ T_{regs} were gated within the $CD3^+CD8^+$ gate. The number of $CD8^+$ T_{regs} in the spleen (c) and ULNs (d) from mice injected with dimethyl sulphoxide (DMSO, control) or α-GalCer ($n = 6$–8 each).

spleen (Supporting information, Fig. S3c), ULNs (Supporting information, Fig. S3d), myometrium (Supporting information, Fig. S3e) and decidua (Supporting information, Fig. S3f) 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 (Supporting information, Fig. S3b).

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 labour/birth. Herein, we investigated whether treatment with rosiglitazone restores the effect of iNKT-cell activation on systemic and local T cells prior to preterm labour/birth. Treating α-GalCer-injected mice with rosiglitazone partially increased the number of total T cells (Fig. 10a), $CD4^+$ T cells (Fig. 10b) and $CD8^+$ T cells (Fig. 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 (Fig. 10d–f). Further, we investigated whether treatment with rosiglitazone restored the number of $CD4^+$ T_{regs} in the decidua. Treating α-GalCer-injected mice with rosiglitazone did not restore the number of $CD4^+$ T_{regs} in the decidua (Supporting information, Fig. S4) and spleen (data not shown).

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 labour/birth, there was (1) a reduction in the number of total T cells, including $CD4^+$

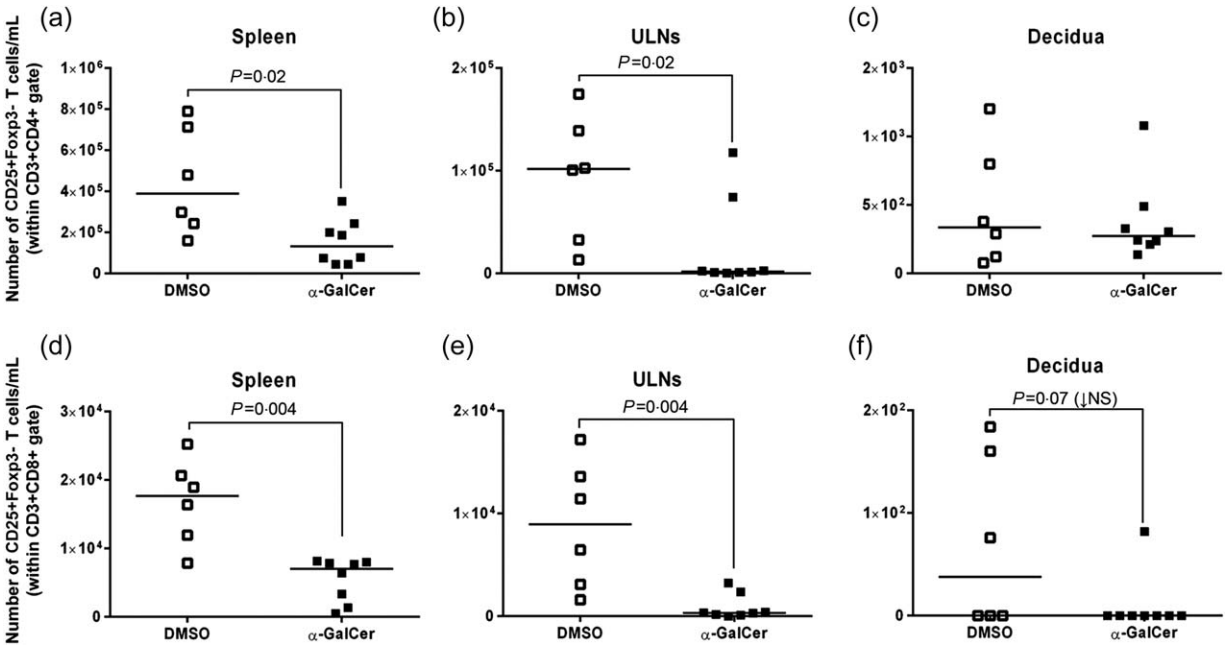

Fig. 9. Invariant natural killer (iNKT)-cell activation via α-galactosylceramide (α-GalCer) causes a reduction of $CD4^+$ and $CD8^+$ forkhead box protein 3 ($Foxp3^-$) responder T cells in the spleen and uterine-draining lymph nodes. Number of $CD4^+Foxp3^-$ responder T cells ($CD3^+CD4^+CD25^+Foxp3^-$ cells) in the spleen (a), uterine-draining lymph nodes (ULNs; b) and decidua (c) from mice injected with dimethyl sulphoxide (DMSO, control) 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 (control) or α-GalCer ($n = 6$–8 each).

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 $CD4^+$ T_{regs} 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 $CD8^+$ T_{regs} 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 partially 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 labour/birth; however, 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 labour/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 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 CD3/TCR complex [51–54]. However, such an interaction initiates signalling pathways simultaneously 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 labour/birth. In line with this concept, we have previously demonstrated that prior to iNKT-cell activation-induced preterm labour/birth, there is an up-regulation 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 up-regulation 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 labour/birth.

In this study, we found that lymphatic and decidual $CD4^+$ T_{regs} were reduced prior to iNKT-cell activation-induced preterm labour/birth. $CD4^+$ T_{regs} 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 [59,60]. During pregnancy, there is an expansion of antigen-specific

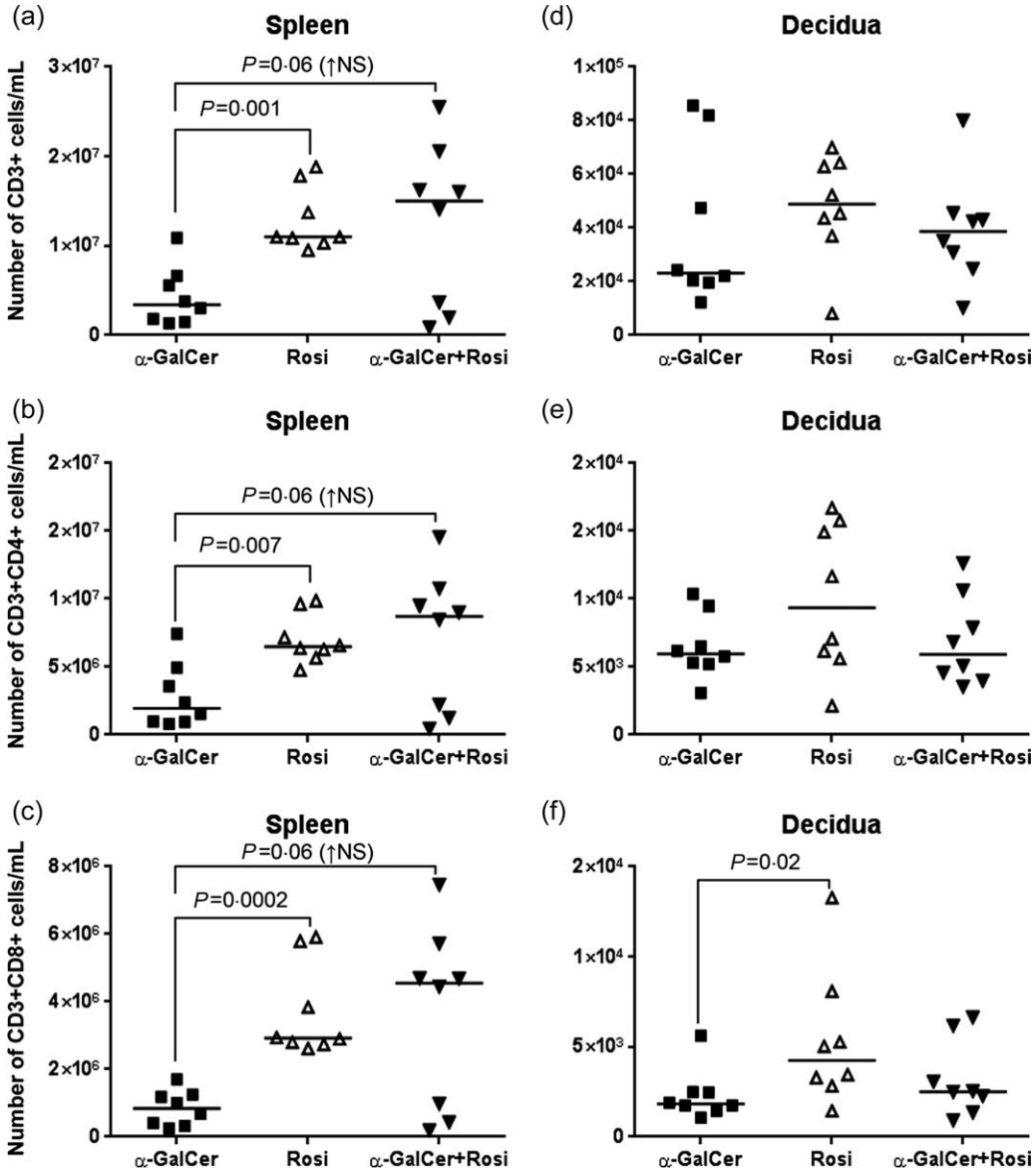

Fig. 10. Treating α-galactosylceramide (α-GalCer)-injected mice with rosiglitazone partially restored the T-cell numbers in the spleen but not in the decidua. Number of T cells (a,d), $CD4^+$ T cells (b,e) and $CD8^+$ T cells (c,f) in the spleen and decidua from mice injected with α-GalCer, rosiglitazone (Rosi) or α-GalCer plus rosiglitazone ($n = 6$–8 each).

$CD4^+$ T_{regs} 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 $CD4^+$ T_{regs} is associated with spontaneous preterm labour [31,32,37,38,65,66]. In addition, a decline in the number of $CD4^+$ T_{regs} at the maternal–fetal interface is observed prior to endotoxin-induced preterm labur/birth [41]. These data support the hypothesis that a breakdown of maternal–fetal tolerance is a mechanism of disease contributing to spontaneous preterm labour [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 $CD4^+$ T_{regs} prior to preterm labour/birth. This hypothesis is supported by the fact that activated iNKT cells regulate $CD4^+$ T_{regs} negatively [67].

Although decidual $CD4^+Foxp3^-$ responder T cells were not altered prior to iNKT-cell activation-induced preterm labour/birth, the number of decidual Th17 cells was increased. The Th17 cell subset is characterized by the expression of IL-17A, IL-17F and IL-22 [68]. The functions of these T cells are wide-ranging, as 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/

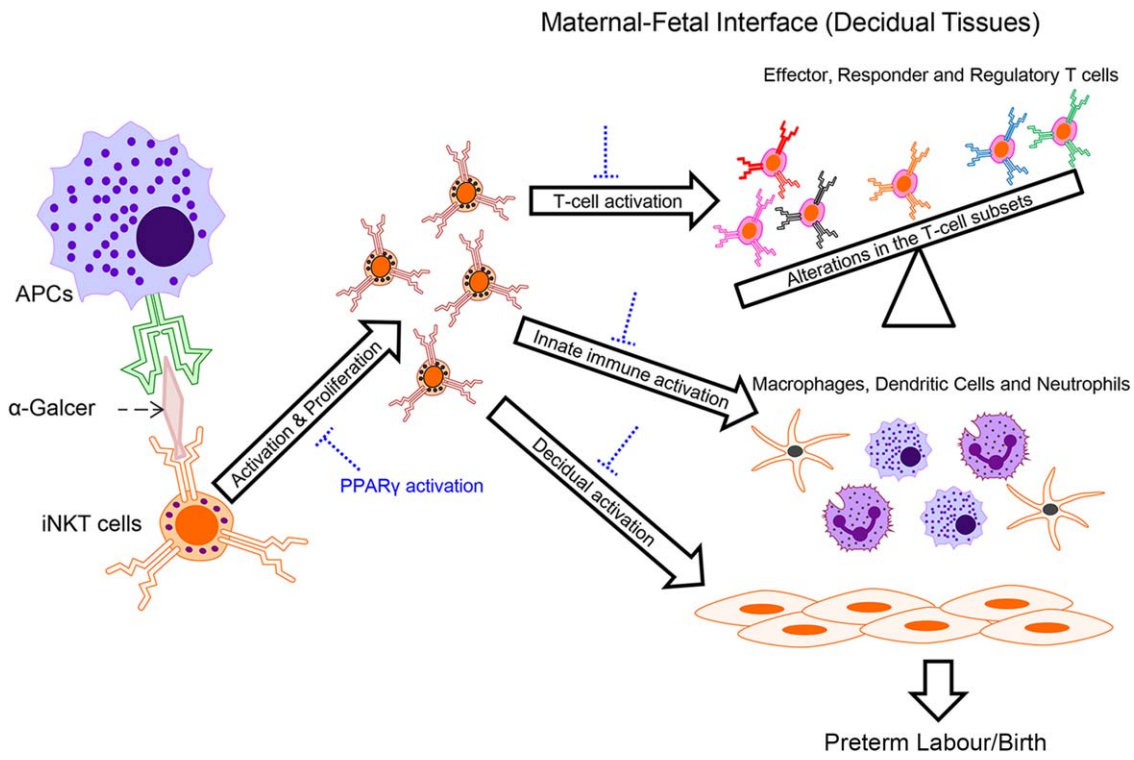

Fig. 11. *In vivo* invariant natural killer (iNKT)-cell activation induces preterm labour/birth by initiating adaptive and innate immune responses. *In vivo* iNKT-cell activation via α-galactosylceramide (α-GalCer) induces T-cell activation (down-regulation of the CD3ε molecule), which translates into alterations in T-cell subsets at the maternal–fetal interface prior to preterm labour/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 labour/birth [19]. Peroxisome proliferator-activated receptor gamma (PPARγ) activation via treatment with rosiglitazone could partially restore such effects and prevent preterm labour/birth.

differentiation. Decidual Th17 cells are also abundant in the chorioamniotic membranes from women who underwent spontaneous preterm labour with acute histologic chorioamnionitis [72]. In fact, an imbalance between effector Th17 cells and $CD4^+$ T_{regs} 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 labour/birth, there is an imbalance between $CD4^+$ T_{regs} and Th17 cells at the maternal–fetal interface.

In addition to altering the number of Th cell subsets, iNKT-cell activation via α-GalCer reduced the number of lymphatic $CD8^+$ T_{regs}. Activated $CD8^+$ T cells expressing Foxp3 share phenotypical and functional characteristics with classical $CD4^+$ T_{regs} [74]. These T cells inhibit T-cell responses (e.g. Th17 cells) *in vivo* [75]. In late pregnancy, an expansion of peripheral and decidual $CD8^+$ T_{regs} was observed when IL-6 null mice ($Il6^{-/-}$) received recombinant IL-6 in order to restore the timing of parturition [34]. Altogether, these data suggest that $CD8^+$ T_{regs} can regulate the timing of parturition and that a reduction in the number of these cells may be associated with preterm labour/birth.

Lastly, we showed that treating α-GalCer-injected mice with rosiglitazone, which prevents iNKT-cell activation-induced preterm labour/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; however, treatment with rosiglitazone restored such effects by activating the PPARγ pathway and preventing preterm labour/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. down-regulation of the CD3/TCR complex). However, treating α-GalCer-injected mice with rosiglitazone did not restore the number of T cells, including $CD4^+$ T_{regs}, 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 fully prevent iNKT-cell activation-induced preterm labour/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 $CD4^+$ T_{regs} in the decidua and prevents preterm labour/birth [78].

In summary, this study 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 labour/birth; however, treatment with rosiglitazone partially restored such effects (Fig. 11). Previously, we have 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 labour and birth, which is also attenuated upon treatment with rosiglitazone [19] (Fig. 11). Together, these findings provide evidence that both innate and adaptive immune cells are implicated in the pathogenesis of preterm labour and that PPARγ activation can represent a strategy for the prevention of this syndrome (Fig. 11). However, a combination of therapeutic approaches may be required to prevent preterm labour/birth entirely and to improve adverse neonatal outcomes.

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Author contributions

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Disclosure

The authors declare no potential conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Gating strategy used to determine viable T cells in the decidua and uterine-draining lymph nodes (ULNs). Lymphocytes were gated using the forward (FSC) and side-scatter (SSC) parameters. Next, viable cells (negative cells for the live/dead dye) were gated within the lymphocyte gate. Lastly, T cells were determined by the expression of CD3ε. The red histogram represents the autofluorescence control and the blue histogram represents the fluorescence signal from the viability dye or the anti-CD3ε antibody.
Fig. S2. Administration of α-galactosylceramide (α-GalCer) did not cause cell death in decidual cells. Number of viable cells in the decidua from mice injected with dimethyl sulphoxide (DMSO, control) or α-GalCer (n = 6–8 each).
Fig. S3. Invariant natural killer (iNKT)-cell activation via α-galactosylceramide (α-GalCer) causes a reduction of double-negative T cells. The gating strategy used to determine double-negative T cells ($CD3^+CD4^-CD8^-$ cells) in the peripheral blood and tissues (a). Number of double-negative T cells in the peripheral blood (b), spleen (c), uterine-draining lymph nodes (ULNs; d), myometrium

(e) and decidua (f) from mice injected with dimethyl sulphoxide (DMSO, control) or α-GalCer (n = 6–8 each).

Fig. S4. Treating α-galactosylceramide (α-GalCer)-injected mice with rosiglitazone did not restore the number of $CD4^+$ regulatory T cells (T_{regs}) in the decidua. Number of $CD4^+$ T_{regs} in the decidua from mice injected with dimethyl sulphoxide (DMSO, control), α-GalCer, rosiglitazone (Rosi) or α-GalCer plus rosiglitazone (n = 6–8 each).