Cutting Edge: Committed Th1 CD4$^+$ T Cell Differentiation Blocks Pregnancy-Induced FoXP3 Expression with Antigen-Specific Fetal Loss

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Pregnancy stimulates induced Foxp3 expression among maternal CD4⁺ T cells with fetal specificity. Although sustained maternal regulatory CD4⁺ T cell (Treg) expansion is essential for maintaining fetal tolerance during pregnancy, the necessity for Foxp3⁺ cells with fetal specificity remains undefined. In this study, we demonstrate that mitigating Treg differentiation among maternal CD4⁺ T cells with a single surrogate fetal specificity elicits Ag-specific fetal loss. Using recombinant Listeria monocytogenes to prime stably differentiated Th1 CD4⁺ T cells with fetal 1-A²:2W1S55–68 specificity refractory to pregnancy-induced Foxp3 expression, we show that Ag delivery by cytoplasmic L. monocytogenes causes selective loss of 2W1S⁺ offspring through CD4⁺ cell- and IFN-γ-dependent pathways. In contrast, CD4⁺ T cells primed by L. monocytogenes restricted from the cell cytoplasm are markedly more plastic for induced Foxp3 expression, with normal pregnancy outcomes. Thus, committed Th1 polarization blocks pregnancy induced Treg differentiation among maternal CD4⁺ T cells with fetal specificity and triggers Ag-specific fetal loss. The Journal of Immunology, 2014, 192: 000–000.
backcrossed to BALB/c (H-2d) or C57BL/6 mice, were described (19, 22). Expression of the 2W1S transgene was assessed using 2W1S primers: 5'-CCAACTCTTCTTGGCATCTCC-3' and 5'-AGATGCGCATAGTCCTCA-AG-3' (22). For infection, *Listeria monocytogenes* were grown to early log-phase (OD600 0.1), washed, suspended in PBS, and inoculated i.v. at the following dosages: delta actin assembly–inducing protein (ΔACTA) *L. monocytogenes*–expressing 2W1S peptide (Lm-2W1S) (16 CFU), delta listeriolysin O delta phospholipase C (ΔLLOΔPLC) Lm-2W1S (10 CFU), or nonrecombinant ΔACTA *L. monocytogenes* (106 CFU) (23–25). For adoptive transfer, CD4+ T cells from the spleen and lymph nodes were purified by negative selection, and one mouse equivalent of CD45.1+ and CD90.1+ cells at a 1:1 ratio was inoculated i.v. into CD45.2+CD90.2+ recipient mice before mating. For depletion, anti-CD4 (GK1.5) or anti–IFN-γ (XMG1.2) Abs were administered i.p. 1 d prior to mating and weekly thereafter (500 μg/dose). All experiments were performed in accordance with Institutional Animal Care and Use Committee–approved protocols.

**Tetramer staining and enrichment**

Mononuclear cells from the spleen and axillary, brachial, cervical, inguinal, mesenteric, pancreatic, and para-aortic/uterine lymph nodes were collected and enriched with PE-conjugated I-A<sup>d</sup> 2W1S<sub>55–68</sub> tetramer (19, 26), followed by cell surface (CD4, CD44, CD25, CD8, CD11c, CD11b, B220, F4/80), intracellular (IFN-γ, IL-17), or intranuclear (Foxp3, T-bet) staining. For stimulation, PMA (100 ng/ml) and ionomycin (1 μg/ml) were added for 5 h in media supplemented with brefeldin A (22).

**Treg and Th17 differentiation**

For Treg differentiation, purified CD4+ T cells were stimulated with syngeneic APCs, 2W1S<sub>55–68</sub> peptide (10 μM), IL-6 (20 ng/ml), IL-23 (10 ng/ml), and TGF-β (1 to 1.6 μg/ml) at 5 h. For Th17 polarization, CD4+ T cells were stimulated with syngeneic APCs, 2W1S<sub>55–68</sub> peptide (10 μM), IL-6 (20 ng/ml), IL-23 (10 ng/ml), and TGF-β (1 ng/ml) in media supplemented with anti-IFN-γ and anti–IL-4 Abs (10 μg/ml each). Five days after stimulation, Foxp3 expression and cytokine production were analyzed by intranuclear and intracellular staining.

**Statistics**

Differences in the percentage of cells, resorption frequency, and number of pups were analyzed using an unpaired Student t test (two groups) or ANOVA with Dunnett correction for multiple comparisons (more than two groups), with *p* < 0.05 taken as statistical significance.

**Results and Discussion**

*L. monocytogenes* selectively expands non-Treg CD4+ T cells, regardless of cytoplasmic entry

Recombinant strains of the intracellular bacterium *L. monocytogenes* have been widely used to characterize the T cell response to infection. We reported previously that *L. monocytogenes* cytoplasmic entry primes Th1 CD4+ T cell polarization and differentiation stability for cells responsive to Lm-expressed Ag (23). However, the use of monoclonal cells from TCR-transgenic mice with fixed specificity may limit their applicability for Treg differentiation, given the discordance in affinity and TCR repertoire between Foxp3+ and Foxp3–CD4+ T cells (27). These drawbacks are bypassed by using MHC tetramers to identify endogenous CD4+ T cells, allowing a more comprehensive analysis of the cumulative polyclonal response (26). Using this approach, we (24) showed that the recombinant wild-type or attenuated ΔACTA Lm mutant that retains cytoplasmic entry each primes selective expansion of Foxp3+ CD4+ T cells with I-A<sup>d</sup>,2W1S<sub>55–68</sub> specificity (24). Reciprocally, pregnancy sired by 2W1S-expressing males stimulates induced Foxp3 expression and Treg accumulation among CD4+ T cells with the same I-A<sup>d</sup>,2W1S<sub>55–68</sub> specificity (19). Given the sharp discordance in Treg differentiation between *L. monocytogenes* infection and fetal stimulation, we reasoned that committed Th1 differentiation by recombinant *L. monocytogenes* could be exploited to investigate the necessity for Foxp3 induction among maternal CD4+ T cells during pregnancy. As controls for infection and 2W1S<sub>55–68</sub> stimulation, CD4+ T cells primed by *L. monocytogenes* restricted from the cell cytoplasm (ΔLLOΔPLC Lm, monocytogenes), with increased plasticity for differentiation into other effector lineages, were evaluated in parallel (23, 25).

We found that ΔACTA *L. monocytogenes* and ΔLLOΔPLC *L. monocytogenes*, each engineered to express 2W1S<sub>55–68</sub> as a secreted recombinant protein, primed robust accumulation of Foxp3+ CD4+ T cells with 2W1S specificity (Fig. 1A). In turn, the percentage of Foxp3+ Tregs among 2W1S+ CD4+ T cells declined precipitously postinfection with each recombinant *L. monocytogenes* compared with naive controls. These shifts were restricted to CD4+ T cells with *L. monocytogenes* specificity, because Foxp3 expression among 2W1S– CD4+ T cells remained similar in *L. monocytogenes*–infected and control mice (Fig. 1A). Furthermore, 2W1S+ CD4+ T cells primed by ΔACTA *L. monocytogenes* selectively upregulated T-bet and acquired the capacity to produce IFN-γ, whereas these shifts were markedly diminished for 2W1S+ cells stimulated by ΔLLOΔPLC Lm (Fig. 1B). Thus, *L. monocytogenes* primes the selective expansion of Ag-specific non-Treg CD4+ T cells, regardless of cytoplasmic entry, whereas Th1 differentiation requires *L. monocytogenes* cytoplasmic entry.

**Discordant plasticity for Foxp3 expression among CD4+ T cells primed by recombinant *L. monocytogenes***

Next, the capacity for Treg differentiation among 2W1S+ CD4+ T cells primed by each *L. monocytogenes* strain was addressed. We found that TGF-β stimulation, along with 2W1S<sub>55–68</sub> peptide and IL-2, induced Foxp3 expression in a dose-dependent fashion among 2W1S+ CD4+ T cells recovered from both groups of *L. monocytogenes*–infected mice. However, at each TGF-β concentration, Foxp3 expression was significantly reduced for cells from ΔACTA Lm–2W1S–infected
mice compared with ΔLLOΔPLC Lm-2W1S–infected mice (Supplemental Fig. 1A). Similarly, after stimulation under Th17-polarizing conditions, 2W1S+ CD4+ T cells from ΔACTA Lm-2W1S–infected mice compared with ΔLLOΔPLC Lm-2W1S–infected mice produced sharply reduced amounts of IL-17 while retaining robust IFN-γ levels (Supplemental Fig. 1B). Thus, CD4+ T cells primed by ΔACTA L. monocytogenes maintain more stable Th1 commitment, with resiliency against differentiation into Tregs or other effector lineages, whereas CD4+ T cells primed by ΔLLOΔPLC L. monocytogenes have more differentiation plasticity.

To investigate how this discordance dictates Foxp3 expression in vivo, pregnancy-induced Treg differentiation among 2W1S+ CD4+ T cells primed by ΔACTA Lm-2W1S compared with ΔLLOΔPLC Lm-2W1S was evaluated. Sixty days postinfection with each L. monocytogenes strain, virgin female mice were mated with allogeneic 2W1S-expressing males, which transforms 2W1S55–68 into a surrogate fetal Ag (19, 22). Consistent with our recent studies using this mating scheme, ~30% of maternal 2W1S+ CD4+ T cells in control mice without prior infection became Foxp3+ by embryonic day 15.5 (Fig. 2A) (19). In contrast, only ~2% of maternal 2W1S+ CD4+ T cells from mice previously infected with ΔACTA Lm-2W1S were Foxp3+, whereas a majority retained T-bet expression and IFN-γ production (Fig. 2). Comparatively, Foxp3 became more readily induced among 2W1S+ CD4+ T cells from mice previously infected with ΔLLOΔPLC Lm-2W1S, albeit at reduced levels compared with naive mice (Fig. 2). Thus, resiliency against Treg differentiation shown in vitro for CD4+ T cells primed by ΔACTA L. monocytogenes is maintained and becomes more pronounced with pregnancy-induced 2W1S stimulation.

Given the unique activation of immune components after ΔACTA L. monocytogenes infection compared with ΔLLOΔPLC L. monocytogenes infection (23, 25), resiliency against subsequent Foxp3 induction among cells from ΔACTA Lm-2W1S–infected mice could also reflect CD4+ T cell–extrinsic differences, in addition to cell-intrinsic shifts in differentiation stability. To discriminate between these possibilities, Foxp3 expression among CD4+ T cells from ΔACTA Lm-2W1S–infected mice or nonrecombinant ΔACTA L. monocytogenes–infected control mice was evaluated after adoptive transfer into naive recipients that subsequently were mated with 2W1S-expressing males. Mice with discordant expression of the CD45.1/2 and CD90.1/2 congenic markers were used so that each donor cell subset could be discriminated from each other and endogenous recipient cells (Fig. 3). We found that resistance against pregnancy-induced Foxp3 expression among CD4+ T cells from ΔACTA Lm-2W1S–infected mice was sustained after adoptive transfer, whereas induced Foxp3 expression to levels indistinguishable from endogenous naive CD4+ T cells were found among donor cells from mice previously infected with nonrecombinant ΔACTA L. monocytogenes (Fig. 3). Thus, despite considerable active debate on the relative stability of Foxp3+ Tregs (28), resiliency against pregnancy-induced Foxp3 expression downloaded from http://www.jimmunol.org/ by guest on April 12, 2017
expected 50% 2W1S+ pups did not deviate in pregnancies among naive mice (49% [43–55%]; mean [95% confidence interval]) or mice with prior ΔLLOAPLC Lm-2W1S infection (50% [44–56%]). 2W1S+ pups were significantly reduced in pregnancies among mice with prior ΔACTA Lm-2W1S infection (38% [30–46%]); and this decline paralleled a 26% reduction in the number of live 2W1S+ pups (3.3/litter in ΔACTA Lm-2W1S–infected mice compared with 4.6/litter in ΔLLOAPLC Lm-2W1S–infected mice or 4.6/litter in naive control mice) (Fig. 4A). Additionally, no differences in fetal resorption (each group’s background level < 10% for each group) were found at these later pregnancy time points, consistent with the necessity of maternal Tregs for implantation or beginning earlier during allogeneic pregnancy (17).

To investigate whether the selective loss of 2W1S+ offspring in ΔACTA Lm-2W1S–infected mice was caused by impaired CD4+ T cell differentiation, we evaluated the impact of CD4 cell depletion prior to mating on subsequent pregnancy. We found that the reduction in 2W1S+ pups among ΔACTA Lm-2W1S–infected mice became outperformed with CD4 cell depletion; the percentage and number of 2W1S+ offspring (52% [46–58%]; 4.8 pups/litter) each rebounded to levels indistinguishable from naive controls (Fig. 4A). Likewise, neutralizing the Th1 effector cytokine IFN-γ prior to mating also abolished the loss of 2W1S+ offspring in ΔACTA Lm-2W1S–infected mice (54% [48–60%]; 4.6 2W1S+ pups/litter) (Fig. 4A). Thus, CD4+ T cells and IFN-γ are each essential for the protective contribution of maternal Tregs with fetal specificity; these findings in mice reinforce the protective role of human decidual Tregs that suppress effector T cell IFN-γ production (9–12). Together, these results suggest that maternal Tregs with fetal specificity confer protection by mitigating activation of Th1 effector cells that are harmful to pregnancy.
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Supplemental Figure 1. Lm cytoplasmic entry primes Th1 CD4+ T cells resilient against subsequent Treg or Th17 differentiation. (A) Representative plots and cumulative data illustrating percent Foxp3 among 2W1S+ CD4+ T cells 60 days after infection with each recombinant Lm, and stimulation with 2W1S55-68 peptide, IL-2 and each concentration of TGF-β for 5 days. (B) IFN-γ and IL-17A production by 2W1S+ CD4+ T cells after stimulation under Th17 polarization conditions for 5 days. Each point reflects the data from a single mouse, and results representative of two independent experiments each with similar results are shown. Bar, mean ± one standard error.