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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.

Materials and Methods

Mice, infection, and adoptive cell transfer

C57BL/6, congenic CD45.1+, and CD90.1+ mice (all H-2b), as well as mice expressing 2W1S55–68 peptide behind the ubiquitously active β-actin promoter interface, which is associated with silencing effector T cell inflammatory responses (9–12). In turn, complementary animal studies allowing for experimental Treg manipulation established that maternal Tregs begin accumulating within the uterine-draining lymph nodes shortly after conception in response to seminal fluid and their necessity for sustaining fetal tolerance during allogeneic pregnancy (13–17). Thus, expanded maternal Tregs protect immunologically foreign fetal tissue from rejection.

With increasingly recognized heterogeneity among Foxp3+ cells, the necessity for unique maternal Treg subsets based on origin and specificity has been proposed (18–20). For example, the accumulation of Foxp3+ CD4+ T cells with specificity to fetal-expressed Ag and fetal resorption induced by prior stimulation with surrogate fetal Ags each suggests that maternal Tregs with fetal specificity play important protective roles (18–21). Induced Foxp3 expression is also likely essential because a majority of maternal Tregs with fetal specificity arise from Foxp3− CD4+ T cells during primary pregnancy, and fetal resorption occurs when peripheral Treg conversion is circumvented in mice with disruption of the foxp3 enhancer conserved noncoding sequence-1 (18, 19). However, despite accumulation of maternal Tregs with fetal specificity, their role in sustaining pregnancy remains uncertain, given the lack of tools for manipulating Tregs in an Ag-specific fashion. To investigate the necessity for maternal Tregs with fetal specificity, pregnancy outcomes were evaluated in mice containing CD4+ T cells, with surrogate fetal specificity stably differentiated into non-Treg effectors prior to mating. Collectively, these studies show that committed Th1 CD4+ T cell differentiation blocks pregnancy-induced Foxp3 expression, causing Ag-specific fetal loss.

The online version of this article contains supplemental material.

Abbreviations used in this article: ΔACTA, delta actin assembly–inducing protein; ΔLLOΔPLC, delta listeriolysin O delta phospholipase C; Lm-2W1S, L. monocytogenes–expressing 2W1S peptide; Treg, regulatory CD4+ T cell.

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backcrossed to BALB/c (H-2d) or C57BL/6 mice, were described (19, 22). Expression of the 2W1S transgene was screened using 2W1S primers: 5′−CCAACTCTGCTGGCATCTCC−3′ and 5′−ATGATGCGCATAGCTCCAG−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 doses: delta actin as the inducing protein (ΔACTA) *L. monocytogenes*—expressing 2W1S peptide (Lm-2W1S) (10^6 CFU), delta listeriolysin O delta phospholipase C (ΔLOΔPLC) Lm-2W1S (10^6 CFU), or non-recombinant *L. monocytogenes* (10^6 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 determination, PMA (100 ng/ml) and ionomycin (1 μg/ml) were added for 5 h in media supplemented with brefeldin A (22).

**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 β 2W1S55–68 tetramer (19, 26), followed by cell surface (CD4, CD44, CD25, CD8, CD11b, CD11c, 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, 2W1S55–68 peptide (10 μM), IL-2 (20 ng/ml), and TGF-β (up to 1.6 ng/ml). For Th17 polarization, CD4+ T cells were stimulated with syngeneic APCs, 2W1S55–68 peptide (10 μM), IL-2 (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**

*Listeria 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 repertoires 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 β 2W1S55–68 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 β 2W1S55–68 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 2W1S55–68 stimulation, CD4+ T cells primed by *L. monocytogenes* restricted from the cell cytoplasm (ΔLOΔPLC *L. monocytogenes*), with increased plasticity for differentiation into other effector lineages, were evaluated in parallel (23, 25).

We found that ΔACTA *L. monocytogenes* and ΔLOΔPLC *L. monocytogenes*, each engineered to express 2W1S55–68, 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 ΔLOΔPLC *L. monocytogenes* (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 2W1S55–68 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.

![Figure 1](http://www.jimmunol.org/Downloadedfrom)
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 allogenic 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 even 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 among Th1 effector CD4+ T cells primed by ΔACTA Lm-2W1S reflects differentiation stability that is both cell intrinsic and Ag specific.

Resiliency against Treg differentiation causes Ag-specific fetal loss

Having established resiliency against Foxp3 induction among 2W1S+ CD4+ T cells after ΔACTA Lm-2W1S infection and the efficiency with which pregnancy-induced 2W1S stimulation primes accumulation of maternal Tregs with the same specificity, these conditions were combined sequentially to address the necessity for Treg differentiation among maternal CD4+ T cells with fetal specificity during pregnancy. Using males heterozygous for the 2W1S transgene for mating, a reduction in the expected 1:1 ratio of 2W1S+/2W1S− pups would illustrate Ag-specific fetal loss. Remarkably, the percentage and number of 2W1S+ pups by embryonic day 15.5 were significantly reduced in mice previously infected with ΔACTA Lm-2W1S compared with ΔLLOΔPLC Lm-2W1S or no-infection controls (Fig. 4A). In particular, although the

**FIGURE 2.** Cytosplasmic L. monocytogenes mitigates accumulation of maternal Tregs with fetal specificity in subsequent pregnancy. (A) Representative plots and cumulative data illustrating the percentage of Foxp3+ cells among maternal 2W1S+ CD4+ T cells during pregnancy (embryonic day 15.5) sired by 2W1S-expressing males among female mice primed by each recombinant L. monocytogenes 60 d prior to mating. (B) T-bet expression and IFN-γ production by 2W1S+ (bold line) and 2W1S− (shaded graph) CD4+ T cells for each group of mice described in (A). Each symbol reflects the data from a single mouse, and results representative of three independent experiments, each with similar results, are shown. Bars are mean ± 1 SE (lower panels).

**FIGURE 3.** Resiliency against Foxp3 expression among CD4+ T cells after L. monocytogenes infection is cell intrinsic and Ag specific. Schematic diagram illustrating the strategy used to discriminate each donor and recipient cell subset, and the percentage of Foxp3+ cells among each group of 2W1S+ CD4+ T cells during pregnancy (embryonic day 15.5) after mating with 2W1S-expressing males. Each symbol reflects the data from a single mouse, and results representative of two independent experiments, each with similar results, are shown. Bars are mean ± 1 SE (right panel).
L. monocytogenes

Together, these results suggest that maternal Tregs suppress effector T cell IFN-γ production; the percentage and number of 2W1S + offspring was, on average, only slightly more than one pup per litter, despite overwhelming resiliency against Foxp3 induction among maternal CD4+ T cells with this specificity. It is important to highlight that this significant loss of offspring reflects circumvented Foxp3 induction among maternal CD4+ T cells of only a single surrogate fetal specificity that may be compensated by Tregs with specificity to other maternal–paternal mismatch Ags in alloimmune pregnancy or, alternatively, Tregs with self-specificity (20). To discriminate between these possibilities, outcomes after syngeneic pregnancy in mice previously infected with ACTA Lm-2W1S and sired by 2W1S-expressing males on the C57BL/6 background were evaluated. Interestingly, the loss of 2W1S+ pups observed in alloimmune pregnancy did not occur in syngeneic pregnancy, where the percentage and number of 2W1S+ pups were indistinguishable between ACTA Lm-2W1S–infected mice and control mice (49% [42–55%] and 4.4 2W1S+ pups/litter after ACTA Lm-2W1S infection compared with 51% [44–57%] and 4.5 2W1S+ pups/litter for naive no-infection controls) (Fig. 4B). Considering the muted protective role of maternal Tregs in maintaining syngeneic pregnancy compared with alloimmune pregnancy (13, 17, 19), these findings are perhaps not unexpected and may be explained by Treg-independent immune-silencing mechanisms recently described during syngeneic pregnancy (30). However, for alloimmune pregnancy that more closely recapitulates the mismatch between MHC haplotype Ags in human pregnancy, the immune-activating properties of fetal MHC alloantigens appear to be needed for uncovering the protective contribution of maternal Tregs with specificity for the surrogate fetal-2W1S minor histocompatibility Ag (1, 2). Applied to the reproductive process in humans and other outbred species in which there is considerably more variability in MHC haplotype and minor histocompatibility Ags between individuals, maternal tolerance likely needs to expand even more to accommodate the enhanced repertoire of foreign paternal–fetal Ags. However, increased antigenic mismatch that stimulates maternal Treg expansion for a broader assortment of foreign fetal Ags may also offset fetal loss shown in this study with resistance against Treg differentiation among maternal CD4+ T cells of a single specificity. These additional questions will require more comprehensive immunological tools capable of tracking CD4+ T cells with specificity for a significantly broader array of fetal and nonfetal specificities. Nevertheless, by experimentally establishing overlap between a single L. monocytogenes–expressed and surrogate fetal Ag, the protective benefits of Treg differentiation among maternal CD4+ T cells with fetal specificity in optimal pregnancy outcomes is revealed.

Disclosures
The authors have no financial conflicts of interest.

References


