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Self-Specific Memory Regulatory T Cells Protect Embryos at Implantation in Mice

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Regulatory T cells (Tregs) play crucial roles in both fetal and tumor development. We recently showed that immunosurveillance by pre-existing CD44^{high}CD62L^{low} activated/memory Tregs (amTregs) specific for self-Ags protects emergent tumor cells in mice. This Treg response of a memory type is more rapid than and dominates the antitumor response of tumor-specific effector T cells. In this study, we report striking similarities between the early Treg responses to embryo and tumor implantation. Tregs are rapidly recruited to uterus-draining lymph nodes and activated in the first days after embryo implantation in both syngeneic and allogeneic matings; express the markers of the amTreg subset; and are at least in part self-Ag specific, as seen in tumor emergence. Unlike in the tumor emergence setting, however, for which preimmunization against tumor Ags is sufficient for complete tumor eradication even in the presence of Tregs, Treg depletion is additionally required for high frequencies of fetus loss after preimmunization against paternal tissue Ags. Thus, amTregs play a major role in protecting embryos in both naive and preimmune settings. This role and the ensuing therapeutic potential are further highlighted by showing that Treg stimulation, directly by low-dose IL-2 or indirectly by Fms-related tyrosine kinase 3 ligand, led to normal pregnancy rates in a spontaneous abortion-prone model. ***The Journal of Immunology*****, 2013, 191: 2273–2281.**

Since Medawar's classic 1953 paper (1), viviparity and especially placental pregnancy have been a riddle for immunologists. Survival of a semiallogeneic conceptus appeared incompatible with self/nonself recognition being a fundamental function of the adaptive immune system. Systemic allospecific or nonspecific immunosuppression cannot be invoked as a stand-alone explanation of maternal–fetal tolerance because even during the first pregnancy the mother is perfectly capable of rejecting paternal strain allografts that are distant (2, 3) or even intrauterine in close proximity to the implantation site (4). These data suggest there is active and specific protection that limits potentially detrimental immune responses while sparing useful ones (5–7).

The maternal immune system thus does sense the presence of the conceptus and mounts an active protective immune response. This task was attributed to suppressor T cells in the late 1970s (8, 9) and more recently to CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Tregs) (10). A Treg deficit severely hampers allopregnancy in nude mice reconstituted with Treg-depleted T lymphocytes (10) or in BALB/c mice treated with an anti-CD25 Ab (11). These and other data in mice (12–15) and humans (16–19) emphasize the importance of Tregs in maintenance of successful allopregnancy.

This role of Tregs in protecting the foreign conceptus is reminiscent of the role of Tregs in protecting tumors, both the conceptus and tumors being proliferating cell masses that are partly self and partly nonself (i.e., possessing paternal or tumor Ags). We recently showed that the immune response at the first encounter between the immune system and emerging tumor cells dictates tumor outcome. Activated/Memory Tregs (amTregs) specific for self-Ags are recruited in the first days after tumor cell emergence in both transplanted and inducible murine cancer models (20). The response of amTregs precedes and pre-empts the slower response of naive conventional T cells with effector potential (effector T cells [Teffs]) that are specific for tumor neo-Ags, and rapidly establishes a dominant tolerant environment (20). We hypothesized that a similar phenomenon could be at work to protect the conceptus in the very early days of its implantation at the blastocyst stage. Thus, we studied the very early regulatory immune responses triggered by embryo implantation and examined the similarities of these responses to the one observed in the context of tumor cell implantation. Striking similarities are indeed observed, notably the early self-Ag–specific driven proliferation of amTregs, which have important therapeutic implications. We report that Tregs boosted by low-dose IL-2 or by Fms-related tyrosine kinase 3 ligand (Flt3-L) can prevent fetal loss in a model of recurrent spontaneous abortion.

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Abbreviations used in this article: amTreg, activated/memory regulatory T cell; DC, dendritic cell; dLN, draining lymph node; dpc, day postcoitum; dpi, day postimplantation; Flt3-L, Fms-related tyrosine kinase 3 ligand; HA, hemagglutinin; Ins, insulin; LN, lymph node; ndLN, non-dLN; PD-L1, programmed cell death ligand 1; pgk, phosphoglycerate kinase; qPCR, quantitative PCR; Teff, effector T cell; Treg, regulatory T cell.

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Materials and Methods

Mice

BALB/c, C57BL/6, female CBA/J, and male DBA/2J mice (6–8 wk old at the initial time of experimentation) were from Charles River, Elevage JANVIER SAS, or Jackson ImmunoResearch Laboratories. Thy1.1 BALB/c congenic mice, insulin (Ins) hemagglutinin (HA) (20, 21), phosphoglycerate kinase (pgk)HA (22), and SFE TCR-HA (23) mice (all in BALB/c background) were bred in our animal facility (ISO9001), in which the mice are kept under specific pathogen-free conditions. Flt3-L$^{-/-}$ mice (C57BL/6 background) were a gift of M. Nussenzweig and were tested in the immunocore of the Rockefeller University animal facility (New York, NY). The rates of CBA × DBA fetal loss in the current study (15–45%) were similar to those in the majority of studies published in the field (24–32). All protocols and treatments either were conducted according to Rockefeller University Animal Care and Use Committee–approved protocols, or were approved by the Charles Darwin Animal Experimentation Ethics Committee of the Centre National de la Recherche Scientifique. To compare experiments conducted in two different animal facilities in the United States and France, we normalized the fetus rejection rates to the mean of the PBS-treated control groups, which was set at 100%.

Visual observation of mating and pregnancy outcome

The sighting of a vaginal plug was denoted as days postcoitum (dpc) 0.5. Female mice were sacrificed from days postimplantation (dpi) 8–12, and the numbers of viable fetuses (F) and resorbed fetuses (R) were recorded visually. Resorbed fetuses are smaller and usually hemorrhagic compared with viable ones. Resorption frequency was calculated as follows: resorption % = R/(R + F).

Tumor experiments

A total of 5×10^5 B16 (melanoma, C57BL/6 background), 4T1, 4T1-HA (breast carcinoma, BALB/c background), AB1, or AB1-HA (mesothelioma, BALB/c background) tumor cells was injected s.c. in the flank of the mice, as described previously (20, 33). Tumor volume was determined by measuring perpendicular tumor diameters L and l using vernier calipers, calculated as (L × l2)/2, and expressed as mm^3. The left inguinal lymph node (LN) was used as the draining LN (dLN). The right inguinal and/or bilateral axillary LNs were used as non-dLNs (ndLNs).

In vivo depletion of CD4$^+$CD25$^+$ T cells

One day before the mating or 1–3 days before tumor injections, female mice received 100 μg anti-CD25 mAb (clone PC61; BD Biosciences) administered by i.p. injection. The anti-CD25 effect on Tregs lasted from 3 to 4 wk at the dose used (11).

IL-2 and recombinant human Flt3-L treatments

IL-2 treatment. Mice were injected i.p. daily with 25,000 IU human rIL-2 (Proleukin; Novartis) for consecutive 10 d, starting 4 d before mating.

Recombinant human Flt3-L treatment. Mice received four s.c. injections of 10 μg recombinant human Flt3-L (Amgen) in 100 μl PBS 3 d apart starting 6 d before mating.

CFSE staining and adoptive transfer of cells

Experiments were performed essentially as described earlier (20, 23). Briefly, 5–10 × 10^6 Thy1.1$^+$ CFSE-labeled unsorted cells from peripheral LNs and spleen from BALB/c mice (used in experiments in Fig. 2), or from SFE TCR-HA transgenic mice (used in experiments in Fig. 3A) were transferred i.v. on dpc 3. After adoptive transfer in wild-type hosts under our experimental conditions, donor Tregs represented 0.1% of splenocytes or LN cells (23). Therefore, 1–5 × 10^6 events were acquired for each analysis. The Treg division index based on CFSE decrease was calculated as follows: (division rate in dLNs − division rate in ndLNs)/division rate in ndLNs.

Adoptive transfer of HA-specific naive Teff and HA immunization

A total of 3×10^6 naive Teffs obtained by magnetically depleting CD25$^+$ cells from SFE mice was transferred i.v. to each BALB/c mouse. The day after adoptive transfer, BALB/c mice were immunized s.c. at the base of the tail with the $HA_{126–138}$ peptide in CFA (Sigma-Aldrich) (34). Two months later, these female mice were mated with pgkHA males either directly or after a single depletion of Tregs (as described above). Similarly, SFE mice were immunized, mated, and Treg depleted before mating as BALB/c mice. The mice used in the tumor experiments were similarly immunized and then challenged with tumor cells 2 months later.

Abs and flow cytometry analysis

Para-aortic and brachial LNs were mechanically dissociated in PBS (3% FBS) and stained with FITC- or V500-labeled anti-CD4, allophycocyanin-labeled anti-CD8, PerCP-labeled anti-Thy1.1/CD90.1, biotin-labeled anti-CD62L (all from BD Biosciences), PE-Cy7–labeled anti-CD25, FITC-labeled anti-CD44, PE-labeled anti–programmed cell death ligand 1 (PD-L1), allophycocyanin-labeled anti-CD103 or PE-labeled anti–glucocorticoid-induced TNFR-related protein, allophycocyanin-labeled anti–CTLA-4, and PE-Cy5–labeled anti-ICOS mAbs (all from eBiosciences). The PE-labeled clone 6.5 anti-clonotypic mAb specific to TCR-HA was produced in rats immunized with the soluble TCR (a gift of H. von Boehmer, Dana-Farber Cancer Institute). Intracellular staining with PE- or eFluor 450–labeled anti-Foxp3, allophycocyanin-labeled anti–CTLA-4, and FITC-labeled Ki67 Abs (all from eBioscience) was done using a kit (FJK-16s; eBioscience). Lymphocytes were acquired with a LSR II cytometer (BD Biosciences) and analyzed with FlowJo (Tree Star) software.

TCR-specific quantitative PCR assays

On dpi 6, para-aortic and brachial LNs were harvested from pregnant or nonpregnant female mice, as were pancreatic LNs from InsHA mice and inguinal LNs from 5-d–old 4T1 tumor-bearing mice. Tregs were sorted on a FACSAria cytometer (BD Biosciences) based on the CD4 and CD25. mRNA was prepared using TRIzol reagent (Invitrogen) and phenol chloroform extraction. RT-PCR and quantification of TCR-HA cDNA were done as previously described (20): TCR-HA cDNA in each sample was quantified by real-time quantitative PCT (qPCR; Applied Biosystems). The primers for the first amplification of the TCR-HA gene were forward primer Vβ8.2, 5′-ACAAGGTGGCAGTAACAGGA-3′, and reverse primer Jβ2.1, 5′-CCTCTAGGACGGTGAGTCGTG-3′. For the nested qPCR, the forward primer Vβ8.2 was 5′-AGTTGGCTACCCCCTCTCAGA-3′, the reverse primer was 5′-GGCCGGGGGAGTTATGC-3′, and the probe labeled with fluorescent reporter was 5′-FAM-ATCAGTGTACTTCTGTGCCAGCGGTGG-TAMRA-3′. As an internal control, endogenous mouse HPRT was also amplified, as follows: forward, 5′-CACGTGGGCTCCAGCATT-3′; reverse, 5′-TCA-CCAGTCATTTCTGCCTTT-3′; probe, 5′-FAM-CCAATGGTCGGGC-ACTGCTCAA-TAMRA-3′. Primers and probes were designed with Primer Express software (version 1.5; Applied Biosystems). Real-time PCR was performed twice using TaqMan Universal PCR master mix (Applied Biosystems) with 200 ng equivalent mRNA in the case of nested qPCR or 600 ng equivalent mRNA in the case of classic direct qPCR. The average Ct of the triplicates was used to calculate the fold change relative to positive control cDNA of cells from TCR-HA SFE mice.

Statistics

Statistical significances were evaluated using GraphPad Prism software (GraphPad Software). Data are presented as mean ± SD, unless otherwise indicated. A p value <0.05 was considered statistically significant. For some experimental groups with nonmatching time points, unpaired t tests with Welch's correction were used to compare groups with pooled time points.

Results

Embryo implantation triggers early recruitment of amTregs in uterine dLNs

We investigated the recruitment of CD4$^+$Foxp3$^+$ Tregs in the LNs of pregnant B6-mated BALB/c female mice. We analyzed Treg numbers and proportions in the uterine para-aortic dLNs and brachial ndLNs, on dpi 1–12 (i.e., dpc 5–16). By dpi 4, we observed a significantly augmented proportion of Tregs in the pregnant mice dLNs compared with the ndLNs, or with the control LNs from nonpregnant mice (Fig. 1A). This increase in Treg proportions in pregnant mice corresponded to more than a doubling of their absolute numbers (Supplemental Fig. 1A). The proportion and numbers of Tregs continued to increase by dpi 7, 10, and 12 in the dLNs. By dpi 12, an increase of Tregs was also observed in the ndLNs, albeit smaller (Fig. 1A, 1B, Supplemental Fig. 1A).

We previously identified the CD44^{high}CD62L^{low} Treg subset as self-specific amTregs (20, 23). In the uterus dLNs of allogeneically mated BALB/c females, compared with nonpregnant virgin controls, we observed a specific and continuous increase in

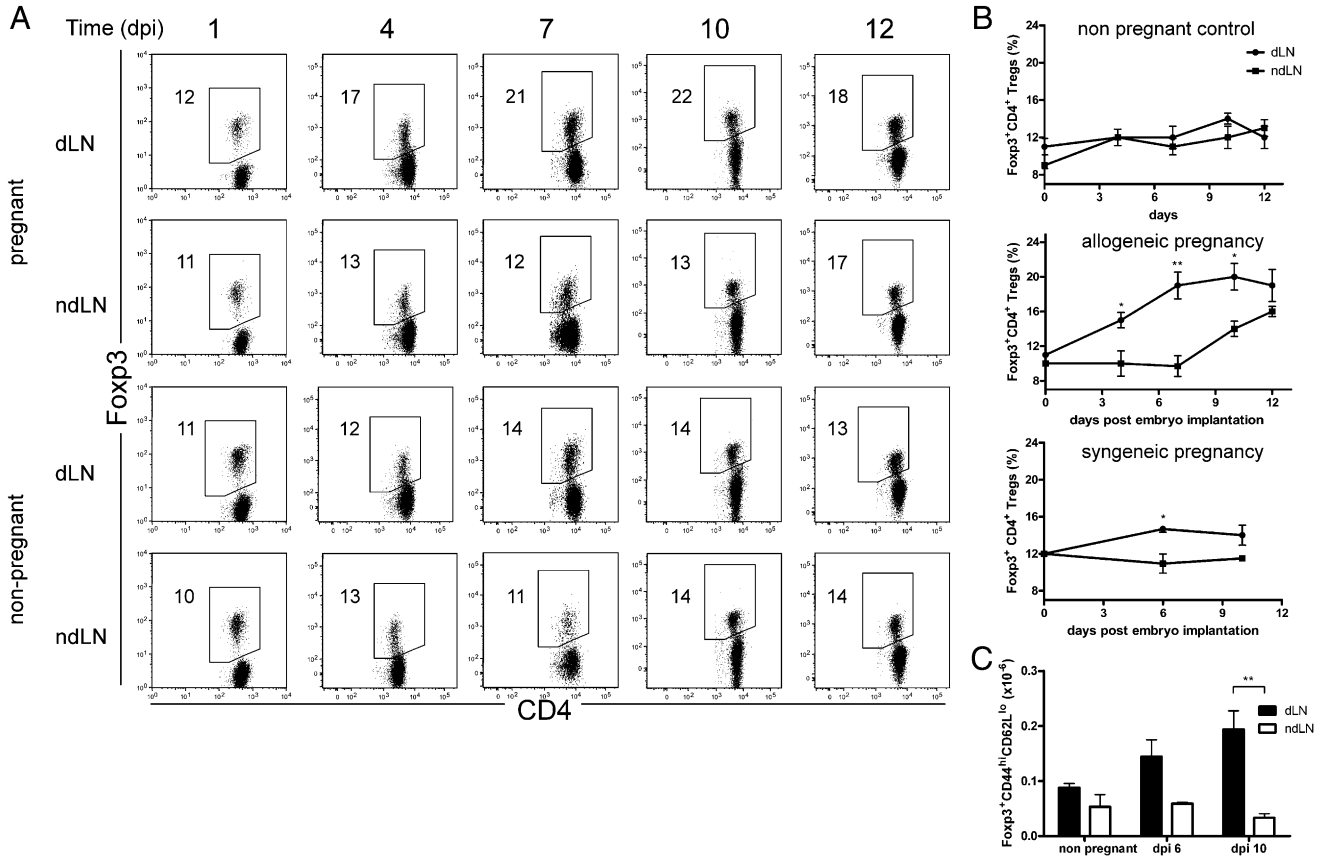

FIGURE 1. Early recruitment of amTregs after embryo implantation. (**A**) Dot plots illustrate the percentages of Foxp3$^+$ CD4$^+$ Tregs in para-aortic dLNs and brachial ndLNs harvested from pregnant B6-mated BALB/c mice on dpi 1, 4, 7, 10, and 12, or from unmanipulated BALB/c female mice. One representative mouse per time point is depicted, n = 3–6 mice per group. Associated statistics are shown in (**B**). (B) Kinetics of Foxp3$^+$ CD4$^+$ Treg frequency in dLNs and ndLNs harvested at the indicated times from the following: nonpregnant female BALB/c mice (*top panel*); B6-mated pregnant BALB/c mice (*middle panel*); and BALB/c-mated pregnant BALB/c mice (*bottom panel*). (**C**) Absolute numbers of CD4$^+$ Foxp3$^+$ CD44^{high} CD62L^{low} amTregs in dLNs and ndLNs harvested from pregnant B6-mated or nonpregnant BALB/c mice at the indicated times. Results are shown in mean ± SEM. n = 3–5 mice per group. Two-tailed unpaired t test significance: $*p \leq 0.05$, $**p \leq 0.01$. Statistics on graph compare each dLN with their respective ndLN. Mice dLNs: pregnant allogeneic versus pregnant syngeneic (Welch's t test with p value = 0.1501); pregnant allogeneic versus nonpregnant (Welch's t test p value = 0.0458; two-way ANOVA p values $<10^{-4}$ for both time and pregnant status).

both number and frequency of amTregs from dpi 6 to dpi 10 (Fig. 1C, Supplemental Fig. 1B). There were no major changes in CD103, CTLA-4, ICOS, PD-L1, CD25, and glucocorticoid-induced TNFR-related protein expression on the recruited Tregs (Supplemental Fig. 1D).

In syngeneic pregnancy (BALB/c-mated BALB/c mice), we also observed an increase of Treg numbers in the dLNs compared with the ndLNs, although to a lesser extent than in allogeneic pregnancy (Fig. 1B, Supplemental Fig. 1C). This suggests that Tregs respond at least in part to self-Ags in the context of pregnancy.

The rapid augmentation of Tregs in the uterus dLNs in pregnancy (B6-mated BALB/c), starting ~3 or 4 d postembryo implantation, was very similar to that observed in an emergent cancer model, 4T1 breast carcinoma cells implanted in BALB/c mice (Supplemental Fig. 1A).

Embryo implantation triggers Treg expansion in uterine dLNs

Next, we studied the proliferation of the different T cell subsets in the dLNs and ndLNs of pregnant mice. Ex vivo CFSE-labeled congenic Thy1.1$^+$ cells were adoptively transferred to B6-mated BALB/c mice on implantation day, and to nonmated BALB/c mice. On dpi 7, 27% of the transferred CD4$^+$Foxp3$^+$ Tregs had undergone at least 1 division, with 9% having divided >6 times (Fig. 2). In contrast, the division rates were approximately half those in ndLNs, and identical to those observed at the steady state in unmanipulated mouse LNs from control nonpregnant mice, the latter reflecting the high turnover of Tregs in a physiological setting (23). The division rates of CD4$^+$Foxp3$^-$ T and CD8$^+$ Teffs in dLNs were low and not significantly different from those in ndLNs and control unmanipulated mouse LNs (Fig. 2). Similar observations were made at dpi 4 and 10 (data not shown).

These data are strikingly similar to those observed in an emergent cancer model, 4T1 breast carcinoma cells implanted in BALB/c mice (Supplemental Fig. 2A), although the magnitude of Treg division is higher in this case. The Treg division index ([division rate in dLNs − division rate in ndLNs]/division rate in ndLNs) increased faster and reached a higher level in the cancer than in the pregnancy setting (Supplemental Fig. 2B).

Treg proliferation during early pregnancy is Ag driven and self specific

We next studied the importance of self-Ags in the recruitment/division of Tregs induced by the conceptus implantation using influenza HA as a model Ag. In InsHA mice, HA is expressed in pancreatic islet β cells under the control of the insulin promoter (20, 21). We mated InsHA female mice with homozygous pgkHA males, in which HA is expressed ubiquitously and from an early embryonic stage by the pgk promoter. HA is thus a self-Ag in both InsHA and pgkHA mice.

We first analyzed the recruitment and division of Tregs obtained from SFE mice that express a HA-specific transgenic TCR (20),

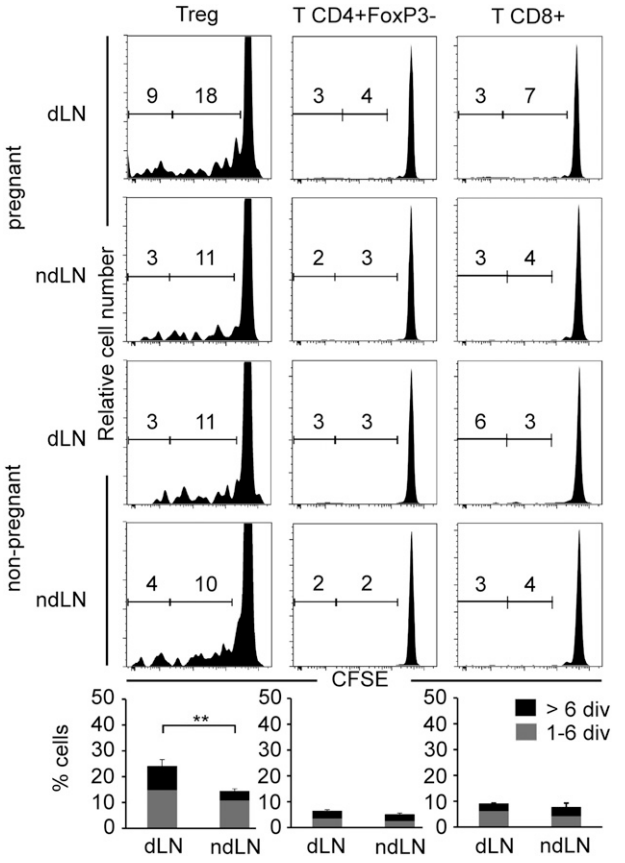

FIGURE 2. Treg division kinetics after embryo implantation. Division profiles of CFSE-labeled Thy1.1$^+$ donor cells in dLNs or ndLNs of embryo-bearing mice. Numbers in each panel represent the percentage of cells having undergone 1–6 divisions (*right*) or >6 divisions (*left*) in B6-mated BALB/c at dpi 7 (*upper panels*) and unmanipulated female BALB/c mice (*lower panels*). Results are from one representative experiment of three independent ones, with panels representing the results of pooled cells from three mice. Bar histogram statistics for Foxp3$^+$ CD4$^+$ Tregs in para-aortic versus brachial LNs from pregnant mice are indicated below. $^{**}p \leq 0.01$.

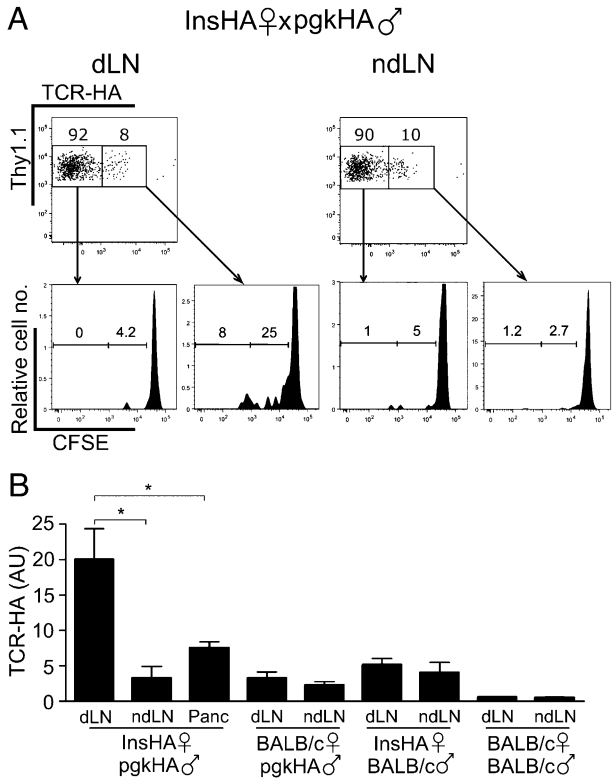

FIGURE 3. Treg proliferation after embryo implantation is self-Ag driven. (**A**) Division of TCR-HA$^+$ and TCR-HA$^-$ donor Tregs in dLNs and ndLNs of pgkHA-mated InsHA females adoptively transferred with CFSE-labeled cells from Thy1.1$^+$ SFE mice transgenic for the anti-HA 6.5 clonotypic TCR. Dot plots show gating strategy of the Thy1.1$^+$/TCR-HA$^+$ and the Thy1.1$^+$/TCR-HA$^-$ among CD4$^+$Foxp3$^+$ cells in dLNs and ndLNs, and histograms below illustrate the level of division of these populations. Cells were transferred on dpc 3 (dpi −1) and analyzed at dpi 3 in pgkHA-mated InsHA mice; one representative experiment of three. (**B**) Natural self-Ag specificity of the non-TCR transgenic endogenous Tregs recruited in dLNs and ndLNs of the indicated pregnant mice was tested by measuring the presence of HA-specific TCR by qPCR (mean ± SEM). Means of three experiments, except for BALB/c × BALB/c combination (two experiments). The *y*-axis represents the arbitrary units of TCR-HA in the indicated LNs of the indicated mating combinations. Two-tailed Mann–Whitney *U* test: $^*p < 0.05$.

adoptively transferred in pgkHA-mated InsHA female mice. At dpi 3, 33% of the CFSE-stained donor TCR-HA$^+$ Tregs had already divided in the dLNs, versus 3.9% in the ndLNs of these InsHA mice (Fig. 3A). In contrast, there was little proliferation of donor TCR-HA$^-$ Tregs (Fig. 3A).

These data are strikingly similar to those obtained after the transfer of CFSE-stained donor TCR-HA$^+$ Tregs in mice implanted with HA-expressing tumors, although in that case the recruitment and division of donor TCR-HA$^+$ Tregs were even more pronounced (20).

Next, we wanted to confirm the recruitment of self-specific Tregs in a wild-type, non-TCR transgenic system. In InsHA mice, HA is expressed in the thymus by an *Aire*-dependent process, and HA-specific TCRs are expressed almost exclusively on Tregs (20). We assessed the recruitment of endogenous natural HA-specific Tregs by quantifying TCRs specific for an immunodominant epitope of HA by qPCR in InsHA mice mated with pgkHA or BALB/c mice (20). Among the various mating combinations tested, we detected the presence of endogenous HA-specific Tregs (i.e., increased qPCR signal of the HA-specific TCR) only in the pancreas and para-aortic dLNs of the pgkHA-mated InsHA females, which are the two main sites where HA Ags are drained in these mice (Fig. 3B).

These results are strikingly similar to those obtained in the setting of cancer where endogenous HA-specific amTregs are recruited only in the HA-expressing tumor itself and in its dLNs (Supplemental Fig. 2C) (20).

Effects of Treg deficit or preimmunization on the fate of embryos

We assessed the effect of Treg ablation in the classic immunological abortion-prone model of CBA/J (H2^k) females mated with DBA/2J (H2^d) males. Treg ablation was achieved by anti-CD25 mAb treatment administered 1 d prior to mating, which led to a Treg deficiency for ∼3–4 wk (11) (Supplemental Fig. 3A). Diphtheria toxin–mediated Treg elimination in mice that express diphtheria toxoid receptors in Tregs could not be used in our setting, because of the different genetic background and also because diphtheria toxin–induced Treg ablation is transient (≤4 d) and incomplete in DEREG mice (35), or leads to rapid and catastrophic lethal autoimmunity in Foxp3^{DTR} mice (36).

By dpi 8, when the animals were sacrificed, examination of the uteri showed similar numbers of implantation sites (whether with viable or resorbing conceptus) in control and Treg-depleted females, indicating that the Ab treatment did not affect fertility. The fetus resorption frequency was increased 2-fold in the Treg-depleted

group, compared with controls (Fig. 4A). These rates were compared with those of BALB/c-mated CBA/J females, a normal pregnancy model, which underwent the same Ab-induced Treg ablation. Fetal resorption frequency in this case was increased 8-fold in the Treg-depleted group, compared with the control group (Fig. 4A).

The quantitative differences in fetal resorption frequencies upon ablation of Tregs in different genetic backgrounds are reminiscent of similar variations observed in different tumor models (20, 37) (Supplemental Fig. 3B).

Next, we investigated whether preimmunization against a single paternal Ag influences pregnancy. We immunized BALB/c mice transferred with $CD4^+CD25^-$ T cells from SFE mice, or SFE mice directly. In these two settings, Teffs contain ~1% or 15–30% of anti-HA–specific cells, respectively, and the HA peptide is not a maternal self-Ag in this context.

These females were then mated with pgkHA males, with or without prior Treg depletion by anti-CD25 mAb. Compared with the 5% basal spontaneous resorption frequency in unmanipulated BALB/c mice, HA-immunized BALB/c had an average of 27% fetal resorption, which increased dramatically to 75% when immunized mice were Treg depleted before mating, and even to 100% in a few cases. A resorption frequency of 48% was observed in the group of HA-immunized SFE females, and this reached 60% when immunization was followed by Treg depletion (Fig. 4B). Collectively, these results show that after HA immunization, resorption of HA-expressing fetuses substantially increased in both BALB/c and SFE females as compared with the resorption level in the naive controls (~5% in both strains). Moreover, the resorption levels did not differ significantly between immunized BALB/c (27%) versus immunized SFE females (48%), despite the difference in the initial number of anti-HA Teffs (1 and 15–30%, respectively).

In contrast, in the cancer setting, preimmunization resulted in a 100% rejection of AB1-HA tumors in BALB/c mice, without requiring Treg ablation (Supplemental Fig. 3C).

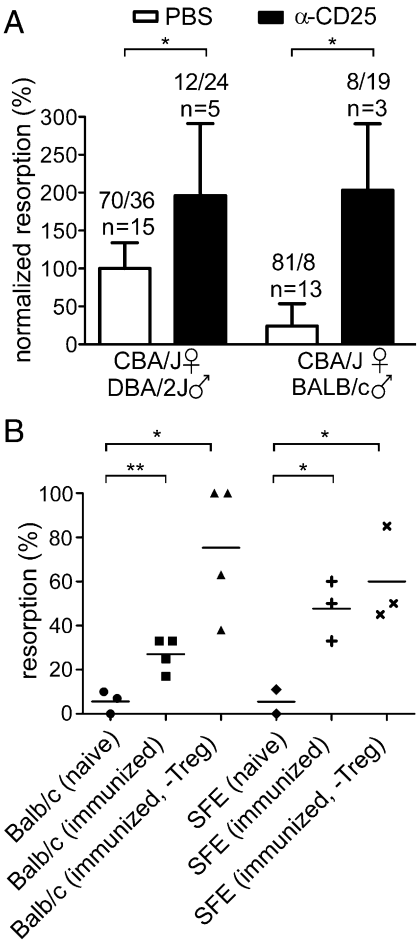

FIGURE 4. Treg ablation and fetal Ag preimmunization impair fetal survival. (**A**) Treg ablation was achieved by anti-CD25 mAb treatment administered 1 d prior to the mating of CBA/J mice. Histograms depict the percentage of fetal resorption in the indicated groups. The results are shown as normalized to the mean resorption frequency of the PBS-treated CBA × DBA control group, which was set at 100%. Numbers above bars indicate the cumulative numbers of resorbed/viable fetuses and the numbers of mice per experimental condition on dpi 8. Two-tailed Mann–Whitney *U* test: $*p < 0.05$. (**B**) Evaluation percentage of fetus resorption after fetal Ag preimmunization of the indicated mice with or without anti-CD25 mAb-induced Treg depletion at the time of the mating. BALB/c female first received anti-HA naive $CD4^+CD25^-$ Teffs from SFE donor mice (i.v.), and were then immunized by HA peptide (s.c., CFA condition). Two months later, HA-immunized SFE-transferred BALB mice, similarly HA-immunized SFE mice and naive SFE and BALB/c female, were mated with pgkHA males directly or after Treg depletion by anti-CD25 treatment (−Treg condition). Viable fetuses or resorption sites were counted on dpi 8 (n = 2–4 mice per group). Two-tailed unpaired *t* test: $*p < 0.05$, $**p < 0.01$.

Treg expansion by Flt3-L or low-dose IL-2 treatments prevents recurrent spontaneous abortion in abortion-prone mice

As Treg depletion increases the frequencies of fetal loss, we investigated whether, on the contrary, Treg induction would reduce the spontaneous abortion rates in the abortion-prone CBA/J × DBA/2J mating model. We previously reported that Treg homeostasis is tightly correlated with the homeostasis of conventional dendritic cells (DCs) (38). Mice deficient in Fms-related tyrosine kinase 3 ligand (Flt3-L) (C57BL/6 background) are genetically deficient in conventional DCs and exhibit a 50% decrease in Tregs compared with syngeneic Flt3-L–sufficient mice (38) (Supplemental Fig. 4A). Compared with B6 × BALB/c matings, Flt3-$L^{-/-}$ × BALB/c matings showed an increase in fetal loss (i.e., visible resorption sites), which did not reach statistical significance (Supplemental Fig. 4B), but resulted in significantly smaller litters (Supplemental Fig. 4C). This suggests a scenario of very early rejection of embryos without detectable resorption sites in this model.

As Flt3-L treatment increases the proliferation of natural Tregs in a DC-dependent manner both at the steady state (38) and also during the pregnancy of DBA/2J-mated CBA/J females (Supplemental Fig. 4D), we evaluated whether Flt3-L treatment could improve the impaired pregnancy in DBA/2J-mated CBA/J females. We observed that the proportion of fetus rejection in CBA/J × DBA/2J matings dropped by 80% after Flt3-L treatment, with 115 viable fetuses versus 20 resorptions in the PBS-treated group and 98 viable fetuses versus 2 resorptions in the Flt3-L–treated group (Fig. 5B).

IL-2 is also known to modulate Treg homeostasis directly by promoting Treg survival, proliferation, and function. For example, we have recently shown that IL-2 administration can cure recent-onset diabetes in NOD mice (39) and significantly induces Tregs and improves clinical symptoms in human hepatitis C virus–induced vasculitis (40). We therefore investigated whether low-dose IL-2 treatment could improve pregnancy outcome in CBA/J × DBA/2J matings. First, we checked the effect of low-dose IL-2 treatment on wild-type CBA/J animals. We observed a statistically significant increase of Tregs in para-aortic and brachial LNs in IL-2–treated versus control mice, which correlated with a higher

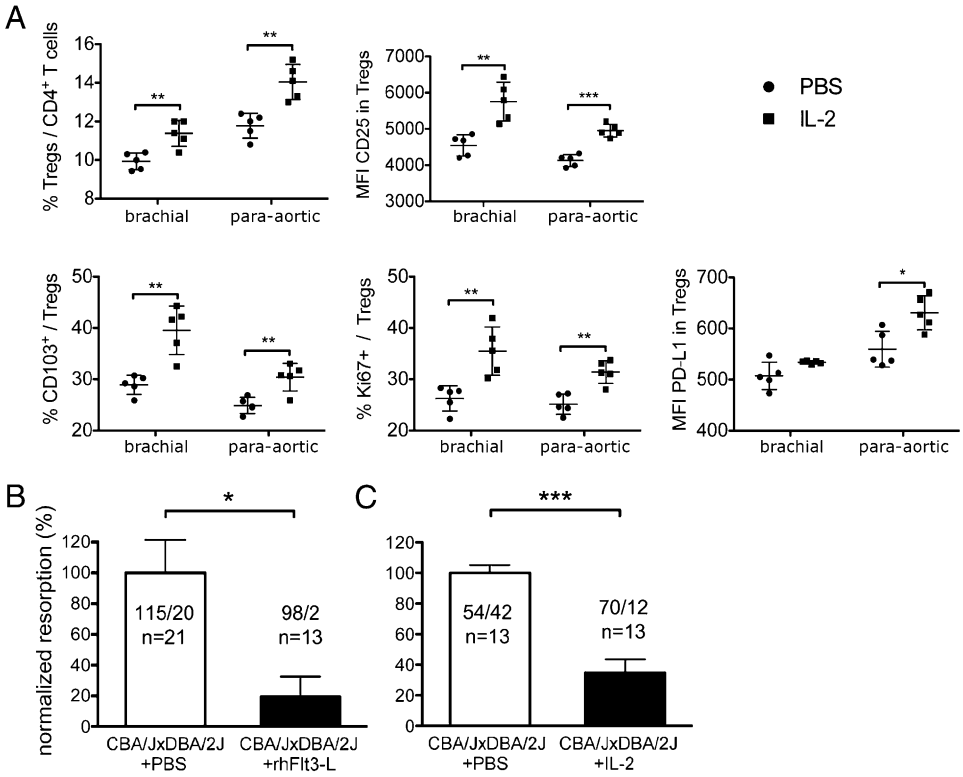

FIGURE 5. Low-dose recombinant human Flt3-L or IL-2 reverse pregnancy loss in a spontaneous abortion-prone model. (**A**) Effects of IL-2 treatment on Treg population of female CBA/J mice. Percentages of CD103- and Ki67-expressing Tregs and CD25 and PD-L1 mean fluorescence index (MFI) of Tregs in para-aortic and brachial LNs after a 10-d treatment with IL-2 ($n = 5$) or PBS ($n = 5$). (**B** and **C**) Effect of recombinant human Flt3-L and IL-2 treatment on the outcome of CBA/J × DBA/2J matings. The results are shown as normalized to the mean resorption frequency of the PBS-treated control groups, which was set at 100%. (B) Viable fetuses and resorption sites were counted on dpi 11, after PBS or recombinant human Flt3-L treatment. In the PBS-treated group, 115 viable fetuses and 20 resorptions ($n = 21$); in the Flt3-L–treated group, 98 viable fetuses and 2 resorptions ($n = 13$); $*p = 0.0123$, two-tailed Mann–Whitney U test. (C) Viable fetuses and resorption sites were counted on dpi 8 post–IL-2 or PBS treatment. In the PBS-treated group, 54 viable fetuses and 42 resorptions ($n = 13$); in the IL-2–treated group, 70 viable fetuses and 12 resorptions ($n = 13$); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, two-tailed Mann–Whitney U test.

proliferation (measured by the expression of $Ki67^+$ cells among Tregs) and increased expression of CD25 and CD103. Tregs were thus activated and expanded systemically in those mice. Notably, we observed an increase of PD-L1 expression by Tregs only in the para-aortic LN of the CBA/J mice.

Next, we evaluated pregnancy outcome in the low-dose IL-2–treated animals. We observed that the proportion of fetus rejection in CBA/J × DBA/2J matings dropped by 65% after IL-2 treatment, with 54 viable fetuses versus 42 resorptions in the PBS-treated group and 70 viable fetuses versus 12 resorptions in the IL-2–treated group (Fig. 5C). It is noteworthy that 4 of 13 IL-2–treated CBA/J females had not a single observable resorption site and all the fetuses were alive. We thus conclude that pregnancy outcome in the abortion-prone CBA/J × DBA/2J mating is substantially improved by low-dose IL-2 treatment.

Discussion

Understanding the mechanisms that protect the allogeneic fetus from attack by the maternal immune system during pregnancy is still a major challenge and has vast heuristic and therapeutic implications in autoimmune diseases and organ transplantation. A large body of findings has demonstrated that fetuses are protected from immune attack in various and redundant ways, with Tregs having a major role. However, relatively little is known about Treg modulation of immune responses at the precise time of embryo implantation, concomitant with the passage from an inflammatory environment (5, 6) to a locally tolerant one. We report in this study that rapid recruitment and activation of pre-existing self-Ag–specific memory Tregs enforce a local tolerogenic environment by outrunning the primary response of Teffs and appear to be a key to embryo survival.

The early requirement for Tregs during pregnancy is supported by the expansion of $CD4^+CD25^+FOXP3^+$ Tregs in the late follicular phase of the menstrual cycle in mice, in preparation for a possible implantation event, followed by a decrease in Treg numbers in the metestrus-disestrus phase as fertility recedes after the window of implantation in a nongravid cycle (41). Women who have experienced recurrent spontaneous abortions have low numbers of Tregs, comparable to numbers observed in postmenopausal women at both the follicular and luteal phases (17, 19), and/or these cells are functionally deficient (42).

Nature of the Ags driving early Treg recruitment/activation

Recently, two groups reported the generation and pivotal role of maternal Tregs specific for paternal alloantigens in successful allopregnancies (14, 43). In particular, Way and colleagues (14) demonstrated that these cells are recruited and actively proliferate starting from midgestation, and later persist as memory Tregs after delivery. They concluded that these cells are important for the success of both primary and secondary pregnancies with males expressing the same alloantigens. However, these data did not shed light on how allogeneic embryos are protected from immune attack at implantation (~day 4) and early gestation, before the appearance of maternal Tregs with paternal specificity at midgestation (~day 11).

In this study, we show that Tregs specific for self-Ags are mobilized very early (3–4 dpi), earlier than the reported mobilization of allospecific Tregs (7.5 dpi) (14), and are essential components of the Treg response to the conceptus. Our results in pgkHA-mated InsHA females unequivocally show that most of the amTreg response at very early gestational stages is self-Ag triggered. This is also supported by our observations and those of others (14) that syngeneic mating triggers an expansion of Tregs in the dLNs. We thus suggest that the recruitment and proliferation of amTregs specific for self-Ags play a crucial role in establishing an early tolerogenic environment that protects the fetus before allospecific Tregs come into play. amTregs are engaged faster and dominate the allospecific Teffs.

Memory alloantigen-specific Tregs (14) are absent at embryo implantation and are thus not crucial in the early establishment of tolerance in primary pregnancies. They may, however, contribute to the tolerant immune response at a later stage as their number increases (14), and could have an important role in secondary pregnancies when the immune attack against the fetus could be more violent due to the presence of allospecific memory Teffs generated during the first pregnancy. In this study, they could reinforce the self-specific amTreg responses for successful fetus protection (14). Tolerant maternal immune responses to the fetus thus appear to be a subtle intercourse of self-specific memory Tregs and allospecific Tregs, the role of which depends on the moment of pregnancy and its primary or secondary status.

Similarities and differences between embryo and tumor handling by Tregs

There are striking similarities in the early T cell response to tumor or embryo implantation, as follows: the response 1) is that of $CD44^{high}CD62^{low}$ amTregs; 2) is driven by self-Ags; 3) is detectable in the first 3–4 d after implantation, preceding the response of Teffs; 4) depends more on memory status than on relative numbers of the cells; and 5) Treg depletion at an early, but not a late, time point induces embryo or tumor rejection (13, 20). The self-Ag–driven response is the main property shared by the immune reactions to cancer and conceptus, as recently confirmed for the cancer setting (44).

There are also important differences between conceptus and tumor handling by Tregs, as follows. 1) After dpi 10–12, the frequencies and numbers of Tregs in uterine dLNs did not continue to increase, although they remained higher than those in control LNs. In contrast, Treg proliferation continues after dpi 10 in emergent tumor models. This difference could be due to a postimplantation transition from an inflammation-like environment to a Th2-dominant environment, which rises at later gestational stages (45). This latter state may involve other mechanisms for embryo/fetus protection, which act as additional and possibly by then predominant protectors of the conceptus. Hormonal changes at the end of pregnancy could also explain this phenomenon (42, 46). 2) More importantly, preimmunization against a paternal Ag—HA in our experiments—only increased the resorption rate, as has also been seen by others in different experimental models (47, 48), whereas, in marked contrast, HA preimmunization resulted in 100% eradication of HA tumors. This result is noticeable because fetus rejection rates have never been reported to reach 100% consistently, except in the somehow artificial setting of nude mice reconstituted with Treg-depleted T cells (10). In our hands, combining Treg depletion with preimmunization resulted in a marked increase in the fetus resorption rate. 3) Finally, local T cell tolerance to (semi)-allogeneic embryos faces the difficulty that alloimmune responses mobilize many more effector cells than Ag-specific responses do. To control these effectors, one could speculate that it would be necessary to engage a large enough number of suppressor cells and/or to build a resistant environment before Teffs could become active and deleterious. The rapid intervention of amTregs, which are quickly put into action because of their memory nature, fulfills these two requirements (number and speed) and appears to be a major component of the early protective responses to embryo implantation in mice. Altogether, our results point to the importance of Tregs in protecting the growing fetus, but also highlight the fact that other mechanisms come into play most likely to ensure at multiple levels survival of the embryo and perpetuation of the species.

Treg response to embryo and tumor in evolution

Viviparity and even placentation appear to have developed before Ag-specific adaptive cellular immunity. Placentae are already found in invertebrates such as onychophora (49), whose representatives have existed since >500 million years, as well as in jawed vertebrates (50). T lymphocytes expressing variable immune receptors that interact with Ags presented by MHC molecules were first found in jawed vertebrates (51). The development of the adaptive immune system in viviparous species thus called for concomitant selection of mechanisms for robust tolerance to embryo implantation and development. Functional Tregs are already found in Tetraodon (−400 million years), in which their depletion produced an enhanced MLR in vitro and inflammation of the intestine in vivo (52). Also, Foxp3 from zebrafish is able to confer suppressive activity on murine T lymphocytes (53). Thus, several lines of evidence suggest that Tregs have played a significant role in implementing maternal–fetal tolerance during the development of adaptive immunity. Induced Tregs that emerged in evolution concomitantly with placentation (15) have also been found to play a significant role in maternal–fetal tolerance (43).

The similarities in embryo and tumor handling by the maternal/host system are not unexpected if one considers analogies between pregnancy and cancer (reviewed in Ref. 54). First, embryo and tumor implantations correspond to the development of highly invasive cells that penetrate/migrate through normal structures to establish their own nutrient supplies. Second, semiallogeneic fetal cells and cancer cells are characterized as being partly self to the mother/patient. That similar responses should be mounted to similar challenges seems logical, but one can then wonder how an immune system that protects deadly tumor cells survived evolution. We speculate that this type of response has been positively selected to protect allogeneic fetuses against immune rejection, in the case of placental or histotrophic viviparity, while developing an adaptive immune system, which appeared after such a reproductive strategy, was first developed. It was not counterselected because cancer development is usually a late-in-life process (55) that usually does not affect reproductive life span. Tregs were probably selected in part to protect allogeneic fetuses against immune rejection, but protection of cancer cells by Tregs became the price paid for an efficient protection of embryos.

Therapeutic implications

Tregs accumulate within the decidua during early human pregnancy (42). Circulating Tregs peak in the second trimester, and decrease to a prepregnancy level after delivery (56). Moreover, recently discovered fetal Tregs in humans (18) appear to limit immune reactions in the fetal tissues (57). Several studies have suggested a close correlation between lower numbers of Tregs and/or reduced expression of Foxp3 and pregnancy failure such as recurrent spontaneous abortions (17, 42, 58–60), in which Treg numbers in the decidua and peripheral blood decrease compared with normal pregnancy levels and reach nonpregnancy levels in

the blood (60). In such patients, Tregs are also qualitatively hampered (42). Altogether, these data suggest that an increase in Treg number and/or function could be therapeutic in pregnancy-related diseases. Therapeutics that boost Treg number and function therefore hold promise.

There is much evidence showing that low-dose IL-2 administration induces Tregs. For example, 5 d of low-dose IL-2 cured recent-onset diabetes in ~40% of treated mice (39). We therefore investigated whether IL-2 could improve the poor fetal survival in the classic murine abortion model, the CBA × DBA/2 system (3). Boosting Treg number did indeed lower the high abortion frequency to a level that is comparable to what is seen in normal, nonabortion-prone mating combinations. However, these data conflict with reports showing that injection of IL-2 in pregnant mice is abortifacient (61, 62). We previously reported a high abortion rate after 2000 IU in three repeated i.p. injections at days 6.5, 8.5, and 10.5 (63). There are several possible, not necessarily mutually exclusive, explanations, such as quantity of IL-2 used (higher doses may recruit CD25-low Teffs), contamination by LPS of crude IL-2 preparations, and/or different injection schedules. In addition, our data obtained with low-dose IL-2 are fully supported by the results obtained with Flt3-L treatment. Initially known as a DC inducer, Flt3-L also acts as a Treg inducer because DC and Treg homeostasis are closely correlated, the number of Tregs increasing when the number of DCs increases and decreasing when DC number decreases (38). Flt3-L treatment also reduced the high abortion frequency to a level similar to what is seen in normal, nonabortion-prone mating combinations. Moreover, fully consistent results were obtained with Flt3-L$^{-/-}$ mice in which the fetal loss rate was increased.

Our recent clinical trial showed that low-dose IL-2 quite specifically induces Tregs without inducing Teffs, and is very well tolerated in patients with autoimmunity (40). Thus, low-dose IL-2 warrants investigation in recurrent spontaneous abortions and infertility caused by implantation failure.

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Disclosures

The authors have no financial conflicts of interest.

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