Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo

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Regulatory T (T reg) cells exert powerful down-modulatory effects on immune responses, but it is not known how they act in vivo. Using intravital two-photon laser scanning microscopy we determined that, in the absence of T reg cells, the locomotion of autoantigen-specific T cells inside lymph nodes is decreased, and the contacts between T cells and antigen-loaded dendritic cells (DCs) are of longer duration. Thus, T reg cells can exert an early effect on immune responses by attenuating the establishment of stable contacts during priming of naive T cells by DCs.

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Several recent studies have revealed the dynamics of live T cell interactions in explanted intact LNs, or intravitally in the LNs of live mice, using laser-scanning microscopy (1–4). T cell interactions with antigen-presenting DCs in LNs are different from those observed in vitro, and it is becoming increasingly apparent that an intact lymphoid organ structure is fundamental for the development of normal immune responses (2, 5). These studies indicated that in the absence of immunization T cells move extensively within the T cell zones of LNs, and this movement facilitates the encounter of rare antigen-specific T cells with antigen-loaded DCs. Upon antigen encounter, T cells partake in a variable period of dynamic interactions and then arrest their locomotion for several hours before regaining motility by 36 h (2–4, 6).

Another component that may play a role, but has not been studied in vivo thus far, is the regulatory T (T reg) cell compartment. T reg cells are major components of immune responses, down-modulating Th1 or Th2 responses against auto, allo, or foreign antigens (7–10). Despite their prominent role, little is known about how T reg cells control immune responses in vivo. In this study, we used intravital two-photon laser-scanning microscopy to visualize the behavior of autoantigen-specific T cells in the presence or absence of naturally occurring T reg cells in intact LNs. We found that 20 h after immunization with autoantigen-loaded DCs, contacts between T cells and antigen-loaded DCs are of shorter duration in the presence of T reg cells than in their absence, indicating an early effect of T reg cells during immune responses.

The online version of this article contains supplemental material.

RESULTS AND DISCUSSION

Demonstration of T reg cell function in the popliteal LN (PLN)

To assess how T reg cells affected encephalitogenic $CD4^+$ T cells in vivo, we used an experimental system in which the host either contains or lacks T reg cells. The experimental system is based on myelin basic protein (MBP) Ac1-11–specific TCR transgenic mice (11). Despite the high frequency of functional MBP-specific T cells, the hemizygous transgenic T/R^+ mice do not develop experimental autoimmune encephalomyelitis (EAE) because of the presence of T reg cells. Genetic ablation of T reg cells, which can be accomplished by crossing T/R^+ mice to $RAG^{-/-}$ mice, to TCR $\alpha^{-/-}\beta^{-/-}$ mice, or by breeding the TCR transgenes to homozygosity (Tg/Tg), leads to the spontaneous development of EAE (11–13). In this study, we used hemizygous T/R^+ as mice harboring T reg cells and Tg/Tg as mice lacking T reg cells. On a C57BL/10.PL genetic background, EAE spontaneously develops in T reg cell–deficient mice (including Tg/Tg mice) beginning

at ~45 d of age, with 50% EAE incidence reached at 60 d and 100% by 120 d of age (12, 13).

We first characterized the cellularity and organization of the PLN in T/R$^+$ and Tg/Tg mice. At 5 wk old or younger, both T/R$^+$ and Tg/Tg mice harbor an equally high number of MBP-specific T cells, the vast majority of which display naive phenotype (Fig. S1 a, available at http://www.jem.org/cgi/content/full/jem.20050783/DC1; reference 11). T/R$^+$ and Tg/Tg mice also have similar proportions of DC subpopulations (Fig. S1 c). The most striking difference is the presence of Foxp3-expressing T reg cells in T/R$^+$ mice but not Tg/Tg mice (Fig. S1 b). The naive status of MBP-specific T cells in the 5-wk-old T/R$^+$ and Tg/Tg mice (11), despite the presence of MBP in the central nervous system, suggests that DCs in PLNs are not presenting endogenous MBP autoantigen (14). Immunohistology was used to confirm that in both types of recipient mice, PLNs were structurally normal and the transferred CFSE-labeled T cells homed to the T cell areas (Fig. S1 d).

Both T reg cell–deficient and –sufficient MBP-specific TCR transgenic mice undergo fulminant EAE upon immunization with MBP emulsified in complete Freund's adjuvant and pertussis toxin, indicating that T reg cells can be overwhelmed or disengaged by strong inflammatory signals (12). Therefore, we immunized with MBP Ac1-11 in IFA to provide an in situ depot for autoantigen delivery to DCs without overwhelming T reg cells. This was an effective strategy because subcutaneous injection in the foot pad with MBP Ac1-11 in IFA triggered IL-2 production by MBP-specific T cells in the PLNs in the Tg/Tg mice, but not the T/R$^+$ mice, at 20 h (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050783/DC1). Thus, MBP Ac1-11 peptide in IFA triggered an immune response in the PLN that was suppressed by endogenous T reg cells.

Influence of T reg cells on the movement of CD4 effector cells in vivo

We next used two-photon intravital microscopy to study the movement of MBP-specific T cells in the presence or absence of T reg cells. CFSE-labeled MBP-specific CD4 T cells purified from Tg/Tg mice (naive effector precursor T cells) were transferred i.v. into T/R$^+$ or Tg/Tg recipient mice (scheme depicted in Fig. 1 a). PLNs from anesthetized mice were exposed and imaged by two-photon laser-scanning intravital microscopy 20 h after T cell transfer. We analyzed the images to determine the speed and arrest coefficient of MBP-specific T cells under both conditions. The arrest coefficient is the proportion of time in which a T cell does not move (threshold <2 μm). The arrest coefficient is generally low when T cells are not engaged in stable contacts with DCs (6). In the absence of immunization, MBP-specific T cells migrated at a mean speed of 6.72 μm/min in T/R$^+$ mice and 6.34 μm/min in Tg/Tg mice, but this difference was not significant (Fig. 1, b and c, and Videos S1 and S2, available at http://www.jem.org/cgi/content/full/jem.20050783/DC1). The mean arrest coefficients were also similar in the presence

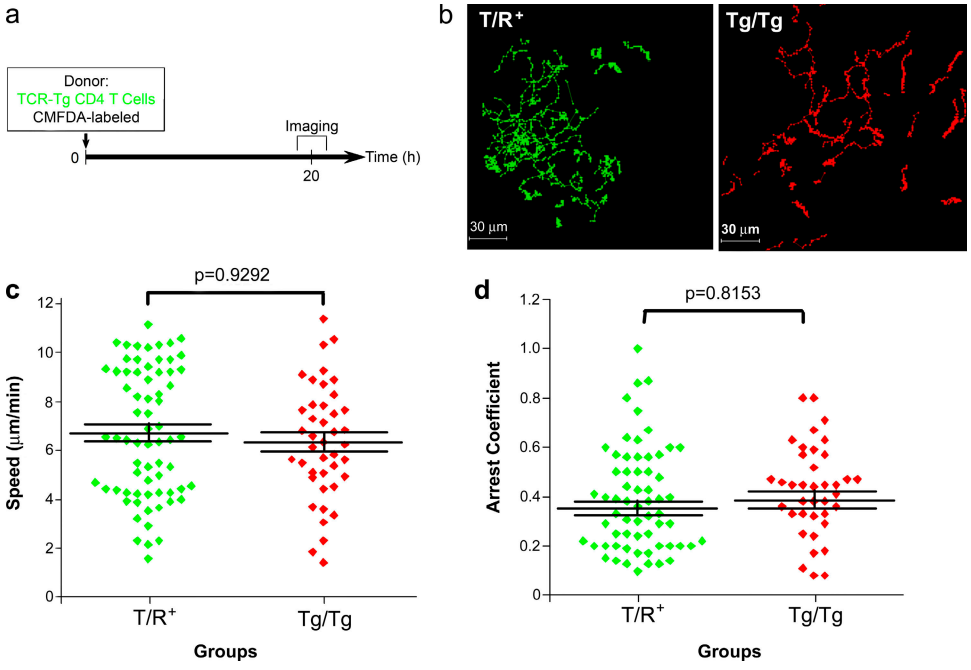

Figure 1. In the absence of immunization, T reg cells do not influence the movement of MBP-specific CD4$^+$ T cells. (a) Experimental protocol. T/R$^+$ or Tg/Tg mice, which have or lack endogenous T reg cells, respectively, received 10^7 CFSE-labeled MBP Ac1-11–specific CD4$^+$ T cells by tail vein injection. 20 h later, PLNs were imaged. (b) Representative tracks of MBP-specific T cells in T/R$^+$ and Tg/Tg recipients. (c) Mean speeds of MBP-specific CD4$^+$ T cells in both types of recipient mice. (d) Arrest coefficient for CD4$^+$ T cells in both types of recipient mice. Results are representative of three independent experiments.

and absence of T reg cells (0.35 and 0.39, Fig. 1 d). Thus, baseline T cell migration is similar in the presence and absence of T reg cells.

We next asked if the effect of autoantigen on MBP-specific $CD4^+$ T cell movement is altered in the presence or absence of T reg cells. Mice were immunized in the hind footpad with MBP Ac1-11 emulsified in IFA, and, at the same time, naive T cells from Tg/Tg mice were transferred i.v. (Fig. 2 a). We then imaged the draining PLNs at 20 h, as this has been shown to be appropriate to observe foreign antigen effects on $CD4^+$ T cells (2, 6, 15). Immunization slowed the MBP-specific T cells substantially only in the Tg/Tg mice (Fig. 2 b and Videos S3 and S4, available at http://www.jem.org/cgi/content/full/jem.20050783/DC1). In the presence of T reg cells, MBP-specific T cells did not significantly change their speed in response to autoantigen, which remained at 6.72 μm/min (Fig. 2 c), but significantly increased their arrest coefficient to 0.49 (Fig. 2 d, $P = 0.0011$). Earlier studies with foreign antigen–specific T cells showed larger effects of antigen on migration in this time frame, but autoantigens are often weaker agonists than foreign antigens, which may account for the smaller effect. In contrast, in the absence of T reg cells, MBP-specific T cells significantly decreased their speed to 3.13 μm/min (Fig. 2 c, $P < 0.0001$) and increased their arrest coefficient to 0.70 (Fig. 2 d, $P < 0.0001$).

To have an internal control for the integrity of the PLN preparations in Tg/Tg mice where antigen-specific T cells sometimes were fully arrested, we cotransferred and imaged WT polyclonal (WT) $CD4^+CD25^-$ T cells from nontransgenic syngeneic chicken β-actin promoter cyan fluorescence protein (CFP) transgenic mice (16). 20 h after transfer, we imaged CFSE-labeled MBP-specific T cells and CFP-expressing WT T cells in the draining PLNs of Tg/Tg mice immunized with MBP Ac1-11 peptide in IFA. The same fields that contained arrested MBP-specific cells had actively moving WT T cells. The latter cells displayed a speed of 5.51 μm/min (Fig. 2 c and Video S5, available at http://www.jem.org/cgi/content/full/jem.20050783/DC1) and an arrest coefficient of 0.50, values that are similar to MBP-specific T cells in unimmunized mice. Thus, we conclude that the presence of T reg cells significantly increased the speed and decreased arrest of antigen-specific $CD4^+$ T cells in the presence of autoantigen.

Interactions between CD4 effector cells and DCs in the presence or absence of T reg cells

One possible explanation for our results could be that in the presence of T reg cells the stable contact between antigen-specific $CD4^+$ naive T cells and DCs was diminished. To directly test this possibility, we transferred CFSE-labeled

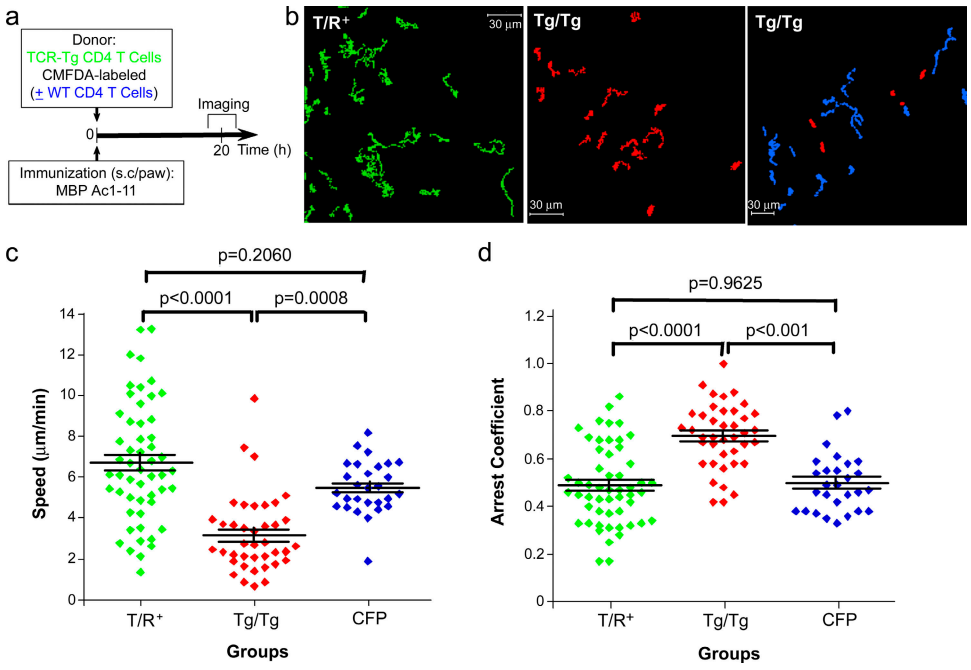

Figure 2. T reg cells release antigen-specific $CD4^+$ T cells in the presence of antigen. (a) Experimental protocol for b–d. T/R^+ or Tg/Tg mice, which have or do not have endogenous T reg cells, respectively, received 10^7 CFSE-labeled MBP Ac1-11–specific $CD4^+$ T cells via the tail vein. As endogenous control, Tg/Tg animals received, by tail vein injection, 1–2 × 10^7 CFSE-labeled MBP-specific T cells from Tg/Tg mice together with 10^7 $CD4^+CD25^-$ T cells from syngeneic CFP-expressing mice. On the same day, mice received 50 μg MBP Ac1-11 peptide emulsified in IFA in the footpad. 20 h after immunization, draining PLNs were imaged. (b) Representative tracks of MBP-specific T cells (green) in T/R^+ and Tg/Tg immunized recipients. (c) Mean speeds of MBP-specific $CD4^+$ T cells in both types of recipient mice. (d) Arrest coefficient for $CD4^+$ T cells in both types of recipient mice. Results are representative of three independent experiments. Representative tracks of WT $CD4^+CD25^-$ T cells (blue) are shown in b. Arrest coefficients for WT $CD4^+$ T cells (blue) are shown in d.

MBP-specific T cells into T/R$^+$ or Tg/Tg animals and, 24 h later, transferred 5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (CMTMR)-labeled antigen-loaded DCs into the foot pad of the same recipient mice (Fig. 3 a). Because the complex between I-A^u and the naturally occurring MBP Ac1-11 is highly unstable, an MBP Ac1-11 mutant peptide (referred to as Ac1-11[4Y]) was used (14, 17). The PLNs were then imaged 20 h later, which allows time for DC migration and therefore should provide similar timing of T–DC interaction as the IFA immunization experiments described above. Antigen-specific T cell movement in PLNs that received antigen-pulsed DCs was qualitatively (Fig. 3 b vs. Fig. 2 b) and quantitatively (Fig. 3 d vs. Fig. 2 d) similar to the movement observed in peptide-immunized animals. Labeling antigen-loaded DCs with a vital dye allowed us to determine the duration of their interaction with MBP-specific T cells in the presence and absence of T reg cells. Contact time was measured by analyzing frame-by-frame individual T cell–DC interactions while viewing the rendered three-dimensional datasets from the most optimal angles. The mean contact time in the absence of T reg cells was 995 s, which was close to the entire time of the datasets, whereas this was reduced to 340 s in the presence of T reg cells (65% reduction, $P = 0.0001$; Fig. 3 c; see also Videos S6 and S7, available at http://www.jem.org/cgi/content/full/jem.20050783/DC1). Thus, the difference in duration of interactions in the presence or absence of T reg cells was about threefold. The arrest coefficient for antigen-specific T cells was significantly lower in the T/R$^+$ recipients compared with the Tg/Tg$^+$ recipients with transferred DCs (36%, $P < 0.0001$; Fig. 3 d). CFP-expressing polyclonal CD4$^+$ T cells used as internal controls had a contact time and arrest coefficient similar to MBP-specific cells transferred to animals that harbor T reg cells (Fig. 3, c and d).

Reduction of CD4$^+$ T cell–DC interaction after adoptive transfer of T reg cells

To exclude other possible differences between T/R$^+$ mice and Tg/Tg mice, we transferred T reg cells into Tg/Tg mice. We previously showed that both CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells from T/R$^+$ mice suppressed EAE development, but later it was found that in T reg cell–deficient experimental systems some of the transferred CD4$^+$CD25$^-$ T cells acquire the CD4$^+$CD25$^+$Foxp3$^+$ T reg cell phenotype and function in vivo, in an IL-2–dependent manner (18, 19). Because this process of peripheral T reg cell induction from CD25$^-$ T cells takes several days, it is possible to examine acute (<36 h) effects of T reg cells by transfer of CD4$^+$CD25$^+$ cells with CD4$^+$CD25$^-$ cells as a negative control. To prove that T reg cells were responsible for the reduction in the duration of contacts between naive antigen-specific CD4$^+$ T cells and antigen-loaded DCs, we transferred purified WT splenic CD4$^+$CD25$^+$ T cells (T reg cells)

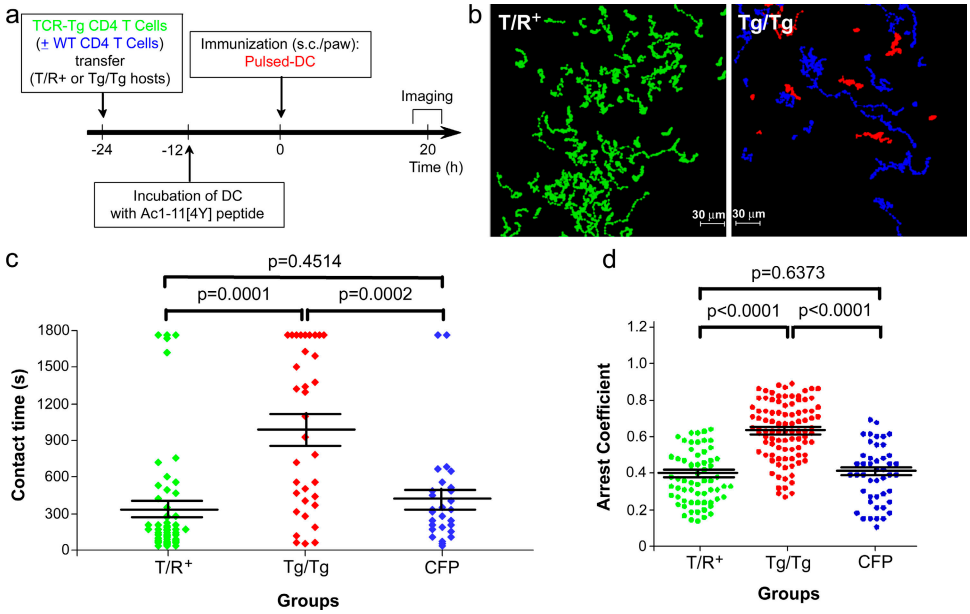

Figure 3. The presence of endogenous T reg cells results in shorter contact time between CD4$^+$ T cells and peptide-pulsed DCs. (a) Experimental protocol. T/R$^+$ or Tg/Tg mice, which have or lack endogenous T reg cells, respectively, received 10^7 CFSE-labeled MBP Ac1-11–specific CD4$^+$ T cells together with 10^7 CD4$^+$CD25$^-$ T cells from syngeneic CFP mice by tail vein injection and, 24 h later, received 5–10 × 10^5 CMTMR-labeled DCs pulsed with MBP Ac1-11[4Y] peptide in the footpad. 20 h after DC transfer, PLNs were imaged. (b) Representative tracks of MBP Ac1-11–specific CD4$^+$ T cells in T/R$^+$ and Tg/Tg DC-transferred recipients. Trackings of CFP-expressing WT CD4 T cells are represented in blue. (c) Contact time between MBP-specific or WT CD4 T cells and antigen-loaded DCs. (d) Arrest coefficient for transgenic (green or red) or WT (blue) CD4$^+$ T cells in T/R$^+$ and Tg/Tg DC-transferred recipients. Results in b–d are representative of three independent experiments.

or control $CD4^+CD25^-$ T cells into Tg/Tg animals and observed the interactions between CFSE-labeled $CD4^+$ naive MBP-specific T cells and CMTMR-labeled Ac1-11[Y4]-loaded DCs 12 h after DC injection (Fig. 4 a). Transfer of T reg cells to Tg/Tg mice recapitulated the qualitative (Fig. 4 b vs. Fig. 2 b and Videos S8 and S9, available at http://www.jem.org/cgi/content/full/jem.20050783/DC1) as well as quantitative (Fig. 4, c and d, vs. Fig. 3, c and d) features of T/R^+ mice. Tg/Tg animals that received T reg cells displayed significantly shorter CD4–DC interaction time compared with Tg/Tg mice that received $CD4^+CD25^-$ T cells (29%, $P = 0.004$). Note that the data shown in Fig. 4 correspond to a 20-min observation experiment (maximum interaction time, 1,200 s). It was not unexpected that the effect of T reg cells transferred into Tg/Tg mice was not as great as the effect of T reg cells endogenously generated in the T/R^+ mice (Fig. 4 c vs. Fig. 3 c) because of issues related to the purification of T reg cells with antibodies, followed by transfer and engraftment. These issues notwithstanding, WT T reg cell transfer into Tg/Tg mice decreased the arrest coefficient of antigen-specific T cells compared with $CD4^+CD25^-$ cell transfer (34%, $P < 0.0001$; Fig. 4 d). Observation at later time points in $CD25^+$ cell–transferred Tg/Tg mice, but not $CD25^-$ cell–transferred mice, showed a decreased number of DCs (not depicted), suggesting that antigen$^+$ DCs may have a shorter half-life in the PLN in the presence of T reg cells, an observation that requires further experimentation.

One of the most sought-after answers in the T reg cell field is to the question of how these powerful cells exert their function in vivo. Our real-time intravital microscopy imaging showed that one of the ways in which T reg cells affect immune responses is by decreasing the contact time between DCs and the precursors of CD4 effector cells. This was observed in two ways, one in which T reg cells were present in the host (T/R^+ mice), and one in which WT T reg cells were transferred into T reg cell–deficient Tg/Tg recipients. Absence of T reg cells always resulted in prolonged, more stable contacts between CD4 cells and DCs. This phenomenon required autoantigen, as T reg cells did not change the movements of MBP-specific T cells in the absence of the autoantigen (Fig. 1). Furthermore, T reg cells did not affect the speed or arrest coefficient of polyclonal $CD4^+$ cells (Fig. 2, c and d). While our paper was under review, a complementary study by Tang et al. (20) obtained similar findings in vitro. Using two-photon imaging of explanted pancreatic LNs in the nonobese diabetic mouse model, they found that the presence of endogenous T reg cells decreased the duration of autoantigen-specific naive and T reg cell interactions with pancreatic islet emigrant DCs. Direct imaging of T reg cells and pancreatic islet emigrant DCs demonstrated the capacity for long-lived interactions of T reg cells with DCs.

An important distinction between T/R^+ mice and T reg cell–transferred Tg/Tg mice is that T/R^+ mice harbor several MBP-specific $CD4^+CD25^+$ T reg cells. In this and other

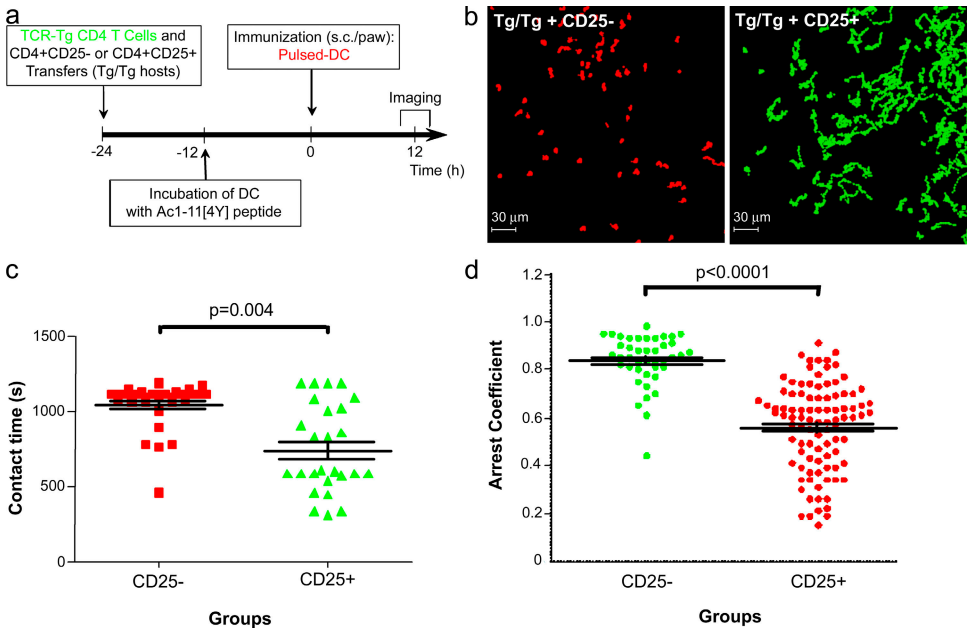

Figure 4. Reconstitution of Tg/Tg animals with T reg cells diminishes the contact time between $CD4^+$ T cells and peptide-pulsed DCs. (a) Experimental protocol. Tg/Tg mice received 10^7 CFSE-labeled MBP-specific $CD4^+$ T cells via tail vein and, on the same day, $5–10 \times 10^5$ $CD4^+CD25^+$ T cells (T reg cells) or $CD4^+CD25^-$ T cells (non–T reg cells) from a syngeneic WT animal in the footpad. 24 h later, CMTMR-labeled DCs pulsed with MBP Ac1-11[4Y] peptide were injected in the footpad, and PLNs were imaged 12 h after DC transfer. (b) Representative tracks of CD4 effector T cells in Tg/Tg animals reconstituted with T reg or non–T reg cells. (c) Contact time between CD4 effector T cells and DCs in Tg/Tg mice that received T reg or non–T reg cells. (d) Arrest coefficient of $CD4^+$ T cells in Tg/Tg mice that received T reg or non–T reg cells. Results in b–d are representative of three independent experiments.

TCR transgenic RAG$^+$ systems, it has been shown that these CD4$^+$CD25$^+$ T reg cells are dual TCR–expressing cells that can be used as antigen-specific T reg cells (21, 22). However, CD4 T cell/DC contact time was also decreased when WT polyclonal CD4$^+$CD25$^+$ T cells were transferred, indicating that uniform MBP Ac1-11 specificity was not required by T reg cells for their function in the model that we studied.

Our intravital data are consistent with previous reports indicating that T reg cells exert their suppressive function by inhibiting DC maturation and/or function (23, 24), although the existence of such a mechanism does not exclude several other means by which T reg cells could function in vivo. The fact that CD4$^+$CD25$^-$ T cells are able to prevent spontaneous EAE—although it required several days before these cells converted to CD4$^+$CD25$^+$Foxp3$^+$ T reg cells in vivo (18)—supports the existence of additional mechanisms that act at later time points.

Thus, our data show an early effect of T reg cells on CD4 T cell responses in vivo. The manner in which T reg cells achieve steady-state regulation of DC interaction with T cells is not clear. Inhibition of arrest is consistent with decreasing the quality of the TCR stop signal or increasing the level of competing chemokinetic signals (25). The intravital approach that we have developed is ideally suited to study this question.

MATERIALS AND METHODS

Mice. MBP Ac1-11–specific TCR transgenic mice (T/R$^+$) have been described previously (11). In brief, genomic TCR-α and -β constructs were coinjected into C57BL/6 zygotes, and the mice were subsequently backcrossed with C57BL/10.PL (The Jackson Laboratory) to incorporate the I.A^u restriction element. Tg/Tg mice are T/R$^+$ mice in which the TCR transgenes have been bred to homozygosity. Tg/Tg mice develop EAE spontaneously due to a defect in T reg cells (12). T/R$^+$ mice always refer to mice hemizygous for the TCR α/β integration. C57BL/6 mice expressing CFP under the transcriptional control of the chicken β-actin promoter and CMV enhancer (16) were purchased from The Jackson Laboratory and crossed to the C57BL/10.PL background for two generations to incorporate the MHC H-2$^{u/u}$. Mice were kept under specific pathogen-free conditions in individually ventilated cages (Thoren) at the Skirball Institute Central Animal Facility, New York University Medical Center. All protocols involving live mice were approved by New York University's Institutional and Animal Care and Use Committee.

Peptides, antibodies, and other reagents. The *N*-acetylated MBP 1–11 peptide (AcASQKRPSQRSK) and the I-A^u–stable Y4 variant (AcASQYRPSQRSK) were purchased from Invitrogen. The anti-TCR clonotyic antibody 3H12 was described previously (13). Other monoclonal antibodies were purchased from BD Biosciences. CFSE and CMTMR were purchased from Invitrogen.

Cell purification, labeling, transfer, immunization protocols, and intracellular IL-2 staining. Naive MBP-specific CD4$^+$ T cells were obtained after negative selection in MACS columns (Miltenyi Biotec), staining splenocytes from Tg/Tg animals with anti-CD8, anti-CD11b, anti-B220, anti-CD11c, and anti-Ter119 FITC-labeled antibodies and incubation with anti-FITC magnetic beads (Miltenyi Biotec). DCs were obtained after positive selection of splenocytes from B10.PL animals using anti-CD11c magnetic beads and columns (Miltenyi Biotec). CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ T cells were obtained from splenocytes of B10.PL animals after a first round of negative selection (as described to obtain naive MBP-specific CD4$^+$ T cells) and a second round of positive selection of CD4$^+$CD25$^+$ cells, staining these CD4 cells with anti-CD25 PE antibody and anti-PE magnetic beads (Miltenyi Biotec). The purities were always ≥90%. Positive selection of CD4$^+$CD25$^+$ cells does not alter their T reg cell activity.

Naive MBP-specific CD4$^+$ T cells from Tg/Tg spleens were labeled for 10 min at 37°C with 5 μM CFSE. 10^7 cells were injected i.v. into recipient T/R$^+$ or Tg/Tg mice via the tail vein. In experiments with immunized animals, shortly after injection of CFSE-labeled CD4$^+$ cells, each animal received subcutaneously in the footpad 20 μl of an emulsion containing 2 mM MBP Ac1-11 peptide diluted in IFA 1:1 (vol/vol). Splenic DCs were purified from syngeneic animals and were pulsed overnight with MBP 10 μM Ac1-11[4Y] peptide, washed, and labeled for 10 min at 37°C with 5 μM CMTMR. These DCs were not subjected to further maturation or activation signals. 5–10 × 10^5 DCs in 20 μl PBS were injected into each footpad of recipient mice 24 h after the labeled CD4 cells. In experiments in which Tg/Tg animals were reconstituted with CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ T cells, 5–10 × 10^5 cells were injected in both footpads of each animal on the same day that they received the MBP-specific CD4$^+$ cells. CFP-expressing WT CD4$^+$ T cells were injected as controls in the relevant experiments. Intracellular staining for IL-2 detection was performed as described previously (19).

Intravital two-photon microscopy data collection and analysis. 20 or 12 h after immunization or DC injection, mice were anaesthetized by an initial intraperitoneal injection of 50 mg/kg ketamine, 10 mg/kg xylazine, and 4 mg/kg acepromazine, and the PLN was prepared microsurgically for intravital microscopy as described previously (2). The prepared LN was immobilized between the edges of two glass slides coated with clay. Each LN was then submerged in normal saline and covered with a glass coverslip. An objective heater (with a temperature probe) was used to maintain and monitor local temperature, which was maintained at 33°C. Care was taken to spare blood vessels. Two-photon imaging was performed in a Bio-Rad Laboratories radiance multiphoton microscope equipped with a 40×, 0.8 numerical aperture objective (Nikon). This microscope was fitted with a Tsunami Ti:Sapphire–pulsed laser (Spectra-Physics) tuned to 800 nm and controlled by LaserSharp2000 software (Bio-Rad Laboratories). All the images were acquired in a depth between 50 and 100 μm. To create time-lapse sequences, we scanned volumes of tissue measuring 50 × 170 × 170 μm (Videos S1–S4) or 50 × 256 × 256 μm (Videos S5–S10) at 4-μm z spacing for 30 min (only the experiment shown in Fig. 4 had a duration of 20 min). To detect CFSE-, CMTMR-, and CFP-labeled cells, 525/50-nm, 620/100-nm, and 510/30–band-pass filters were used, respectively. Image and cell movement analysis was performed with Volocity software (Improvision), which was also used for semi-automated tracking of cell motility. Determination of speeds and arrest coefficients were made using a displacement threshold of 2 μm as described previously (26). Arrest coefficients were calculated as the ratio between the time that a cell was not moving (instantaneous speed <2 μm/min) and the total time the cell was observed. Interactions between T cells and DCs were defined as physical contacts lasting >30 s. Overall, we analyzed 3,820 cell tracks from 46 imaging fields in 24 mice for these studies. Statistical significance was determined using the Mann-Whitney test and ANOVA. p-values <0.01 were considered significant. Each dot plot graphic contains horizontal lines representing mean and standard errors.

Immunofluorescence. PLNs from animals submitted to intravital microscopy or similarly treated were harvested and frozen in Tissue-Tek O.C.T. media (Sakura Finetek), and 8-μm-thick slices were cut. Slides were fixed in 1% paraformaldehyde and blocked with 10% normal mouse serum for 1 h at 24°C. Slides were then stained with the corresponding fluorescent antibodies for another 2 h at 24°C. After washing with PBS, slides were mounted with Fluoromount G mounting media (SouthernBiotech).

Online supplemental material. Fig. S1 illustrates the differences between T/R$^+$ and Tg/Tg mice regarding the presence of T reg cells in the former

mouse type but not the latter, and the overall similarities between the two mouse types in all other lymphocyte compartments, as well as antigen-presenting cell subsets and LN architecture. Fig. S2 shows the difference in IL-2 production in the T/R$^+$ and Tg/Tg mice immunized with MBP Ac1-11 in IFA. The time-lapsed Videos S1 and S2 illustrate MBP Ac1-11–specific CD4$^+$ T cell movement in Tg/Tg or T/R$^+$ PLNs, respectively. Videos S3 and S4 illustrate the same cells transferred to Tg/Tg or T/R$^+$ immunized animals, and Video S5 shows MBP-specific CD4$^+$ T cells (green) and WT CD4$^+$ T cells (blue) in the PLN of an MBP-immunized Tg/Tg animal. Videos S6 and S7 illustrate the interactions between antigen-specific CD4 T cells and antigen-loaded DCs in Tg/Tg or T/R$^+$ animals, and Videos S8 and S9 illustrate the same T–DC interactions in Tg/Tg animals adoptively transferred with CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ T cells.

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