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Cutting Edge: Cure of Colitis by CD4⁺CD25⁺ Regulatory T Cells¹

Christian Mottet,² Holm H. Uhlig,² and Fiona Powrie³

CD4⁺CD25⁺ regulatory T cells have been shown to prevent T cell-mediated immune pathology; however, their ability to ameliorate established inflammation has not been tested. Using the CD4⁺CD45RB^{high} T cell transfer model of inflammatory bowel disease, we show that CD4⁺CD25⁺ but not CD4⁺CD25⁻CD45RB^{low} T cells are able to cure intestinal inflammation. Transfer of CD4⁺CD25⁺ T cells into mice with colitis led to resolution of the lamina propria infiltrate in the intestine and reappearance of normal intestinal architecture. CD4⁺CD25⁺ T cells were found to proliferate in the mesenteric lymph nodes and inflamed colon. They were located between clusters of CD11c⁺ cells and pathogenic T cells and found to be in contact with both cell types. These studies suggest that manipulation of CD4⁺CD25⁺ T cells may be beneficial in the treatment of chronic inflammatory diseases. The Journal of Immunology, 2003, 170: 3939–3943.

The inflammatory bowel diseases (IBD),⁴ which include Crohn's disease and ulcerative colitis, are chronic inflammatory disorders affecting ~0.3% of the Western population (1). Understanding of the pathogenesis of IBD has been aided by the development of animal models that mimic aspects of the human disease (2). A well-characterized model of IBD is the transfer of predominantly naive CD4⁺CD45RB^{high} T cells into syngeneic immunodeficient mice (3). Four weeks post-T cell transfer, mice develop clinical signs of a progressive and chronic IBD (4).

Cotransfer of CD4⁺CD45RB^{low} T cells together with potentially pathogenic CD4⁺CD45RB^{high} T cells prevents development of colitis by mechanisms involving TGF- β and IL-10 (5). Recently, regulatory T (T_R) cells capable of inhibiting colitis were found to enrich within the CD25⁺ subset (6). This subset, which is present in the thymus and the periphery of mice, rats, and humans, has been shown to suppress a number of additional T cell-mediated responses in vitro and in vivo, including autoimmune disease, allograft rejection, and tumor immunity (7–9).

To be of use as therapeutic agents for inflammatory and autoimmune diseases, T_R cells must be able to inhibit ongoing T

cell responses and reverse established pathology. However, to date, CD4⁺CD25⁺ T_R cells have only been shown to prevent immune pathology. In this report, we assess the ability of CD4⁺CD25⁺ T_R cells to reverse established colitis.

Materials and Methods

Mice

BALB/cJ, C57BL/6J, congenic C57BL/6.SJL.CD45, C.B-17 SCID (SCID), and C57BL/6 recombinase-activating gene (*rag*)-1 deficient (*rag1*^{-/-}) mice were bred under specific pathogen-free conditions. All mice used were >7 wk old.

Cell purification and flow cytometry

CD4⁺ T cell subsets were isolated from spleens as described (10). For MACS sorting, CD4⁺-enriched cells were stained with biotinylated anti-CD25 (7D4), followed by streptavidin MACS beads, and sorted on an AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD4⁺CD25⁻ fraction was then stained with anti-CD45RB-FITC (16A), followed by incubation with anti-FITC MACS beads, and the CD4⁺CD25⁻CD45RB^{low} fraction was isolated. For FACS sorting, CD4⁺-enriched cells were stained with anti-CD45RB, anti-CD25, and anti-CD4 (H129.19), and sorted on a MoFlo (Cytomation, Fort Collins, CO). The purity of MACS- and FACS-sorted cells was >90% and >99%, respectively. Because similar results were obtained using MACS or FACS sorting, data were pooled.

T cell transfer experiments

SCID and *rag1*^{-/-} mice were injected i.p. with 4 × 10⁵ syngeneic CD4⁺CD45RB^{high} T cells. Mice developed clinical signs of colitis 3.5–4.5 wk (wk 4) posttransfer. Mice with clinical signs of disease received either 10⁶ CD4⁺CD25⁺ or 10⁶ CD4⁺CD25⁻CD45RB^{low} (CD4⁺CD25⁻) T cells i.p., or no treatment, or were sacrificed to assess the severity of colitis. In some experiments, mice were injected i.p. with 10⁵ CD4⁺CD25⁺ T cells at the same time as CD4⁺CD45RB^{high} cells. Mice were observed daily and weighed weekly. Any mice showing clinical signs of severe disease were sacrificed according to the United Kingdom Animals Scientific Procedures Act of 1986.

Enumeration of CD4⁺ cells

Lymphocyte suspensions were prepared from spleen, mesenteric lymph node (MLN), and colon lamina propria (LP) (1), and analyzed for CD4 (H129.19), TCR- β (H57-597), and CD45.1 (A20) using a FACSCalibur or FACSsort (BD Biosciences, San Jose, CA).

Histology

Tissue sections were stained with H&E as well as alcian blue and periodic acid-Schiff solution (11). Colitis severity was graded semiquantitatively from 0 to 4 in a blinded fashion (6).

For CD4⁺ cell enumeration, tissue samples were snap frozen. Acetone-fixed cryosection slides were blocked with donkey serum (Sigma-Aldrich, Poole,

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⁴ Abbreviations used in this paper: IBD, inflammatory bowel disease; T_R, T regulatory; *rag*, recombinase-activating gene; MLN, mesenteric lymph node; LP, lamina propria; DAPI, 4',6'-diamidino-2-phenylindole; POD, peroxidase.

U.K.) and stained with anti-CD4 (clone RM4-5; BD Biosciences) followed by donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA). The mucosal CD4 density represents the average of four areas per mouse.

For multicolor analysis, sections were sequentially stained for CD4, CD45.1, CD11c, and cell nuclei (4',6'-diamidino-2-phenylindole (DAPI); Sigma-Aldrich). Endogenous peroxidase (POD) activity was inhibited. After CD4 staining and blocking with rat serum, binding of biotinylated anti-CD45.1 (A20; BD Biosciences) was revealed with avidin-POD (Vector Laboratories, Peterborough, U.K.), followed by tyramid-Cy3 amplification (NEN Life Science Products, Zaventem, Belgium). POD activity was blocked, and sections were incubated with hamster anti-CD11c (HL3; BD Biosciences) and donkey anti-hamster POD (Jackson ImmunoResearch), followed by Cy5 tyramide amplification (NEN Life Science Products).

To analyze the proliferative capacity, paraformaldehyde- and methanol-fixed frozen sections were stained for Ki67, CD4, CD45.1, and with DAPI. Ki67 expression was detected using mouse anti-Ki67 (B56) followed by anti-mouse Ig (Jackson ImmunoResearch).

Statistics

Two-tailed Mann-Whitney *U* test and Fisher exact test were performed using GraphPad Prism 3.00 (GraphPad, San Diego, CA). Values of $p \leq 0.05$ were regarded as significant. Data are presented as mean \pm SEM.

Results and Discussion

CD4⁺CD25⁺ T_R cells reverse wasting disease and colitis

To determine whether T_R cells can ameliorate established colitis, we injected immunodeficient mice with clinical signs of colitis with CD4⁺CD25⁺ or CD4⁺CD45RB^{low}CD25⁻ T cells (Fig. 1*a*). Three to 4 wk after CD4⁺CD45RB^{high} T cell transfer, mice develop clinical signs of colitis including piloerection, hunching, anal inflammation, diarrhea, and weight loss. Colitic mice receiving 10⁶ CD4⁺CD25⁺ T cells typically started to recover 2 wk after the transfer with gradual disappearance of clinical signs. In contrast, mice injected with 10⁶ CD4⁺CD25⁻ T cells or control mice without second cell transfer continued to lose weight and did not show signs of clinical improvement (Fig. 1*b*). Overall, 9% of colitic mice injected with CD4⁺CD25⁺ T cells had to be sacrificed during the experimental interval of 14 wk (median time to sacrifice, 14.0 wk), compared with 78% of mice injected with CD4⁺CD25⁻ ($p = 0.004$; median time to sacrifice, 5.7 wk) and 74% of control mice without second cell transfer ($p = 0.002$).

Because wasting disease does not always correlate with colon inflammation, we then investigated histological markers of co-

litis. Colonic architecture in the control mice sacrificed at wk 4 was characterized by a leukocytic infiltrate in the LP, depletion of goblet cells, and a moderate epithelial cell hyperplasia with occasional ulceration corresponding to an average colitis score of 3.1 ± 0.2 (Figs. 1*c* and 2*a*). Compared with this group, mice that received CD4⁺CD25⁺ cells had significantly lower colitis scores (1.5 ± 0.9 ; $p < 0.01$) when sacrificed between 5 and 14 wk. There was restored colonic architecture, denoted by a reduction of epithelial cell hyperplasia (Fig. 2*a*), rare LP infiltrates (Fig. 2, *a* and *b*), and reappearance of goblet cells (Fig. 2*c*). Despite resolution of the inflammatory response, isolated clusters of leukocytes in the LP remained. These clusters were a feature of mice that had resolved colonic inflammation. Similar clusters occurred in lower numbers in the LP of mice that were prevented from developing colitis by injection of CD4⁺CD25⁺ cells at the same time as the CD4⁺CD45RB^{high} population. In mice that received either CD4⁺CD25⁻ cells (colitis score of 3.0 ± 0.7) or control mice receiving no second cell transfer (3.2 ± 0.4), we did not see any histological improvement (Figs. 1*c* and 2).

In accordance with the histology scores, mice that received CD4⁺CD25⁺ cells had a 3-fold reduction in CD4⁺ cell density in the colon in comparison to control mice at wk 4 as well as mice transferred with CD4⁺CD25⁻ cells or untreated mice ($p = 0.028$). After recovery from colitis, mice had a similar CD4⁺ cell density in the colon as mice receiving an initial cotransfer of CD4⁺CD45RB^{high} and CD4⁺CD25⁺ cells (Fig. 2*b*).

Taken together, our results show that a single transfer of T_R cells improves clinical status, survival rate, and intestinal pathology of mice with established colitis. Ten weeks after CD4⁺CD25⁺ T cell transfer, the histological colonic abnormalities were almost completely resolved. The leukocytic infiltrate disappeared with only isolated clusters of CD11c⁺ and CD4⁺ T cells remaining in the LP. Interestingly, similar normalization of the mucosa with remaining leukocytic clusters has been observed in IBD patients after treatment with immunosuppressive or anti-inflammatory drugs (12). Additional experiments are required to determine whether T_R cell therapy will also be effective at resolving inflammation in nonlymphopenic hosts.

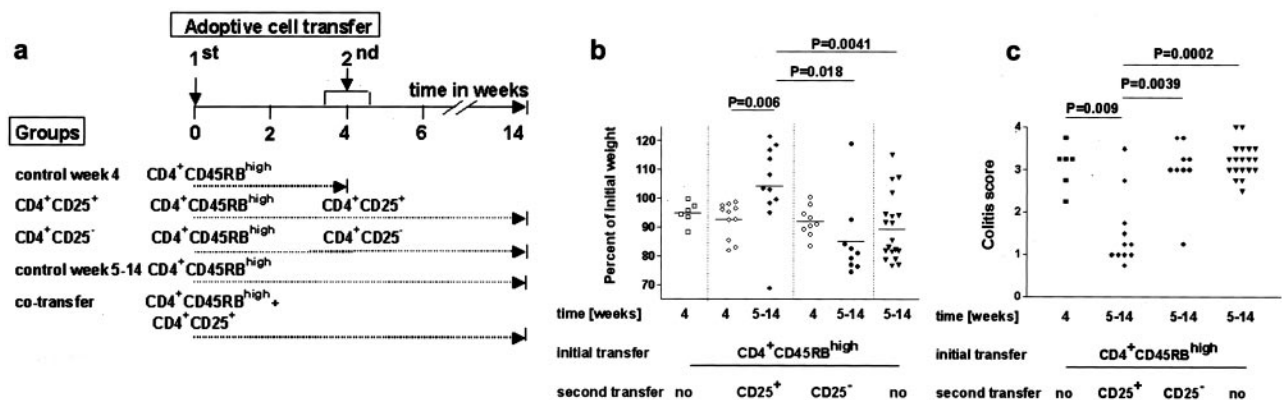


FIGURE 1. Cure of colitis by CD4⁺CD25⁺ T_R cells. *a*, Experimental protocol. Immunodeficient mice received CD4⁺CD45RB^{high} T cells. CD4⁺CD45RB^{low}CD25⁺ or CD4⁺CD45RB^{low}CD25⁻ T cells were transferred at the time points indicated. Mice were monitored for 14 wk, except for the control group, which was sacrificed at wk 4. *b*, CD4⁺CD25⁺ T cells reverse wasting disease. Data represent body weight as a percentage of the initial weight of individual mice. *c*, CD4⁺CD25⁺ T cell transfer ameliorates established colitis. Colitis scores of individual mice are shown. In *b* and *c*, data of two experiments were pooled (SCID and *nag1*^{-/-} recipients). Symbols represent individual mice. Three additional experiments with colitic mice receiving a second CD4⁺CD25⁺ T cell transfer gave similar results.

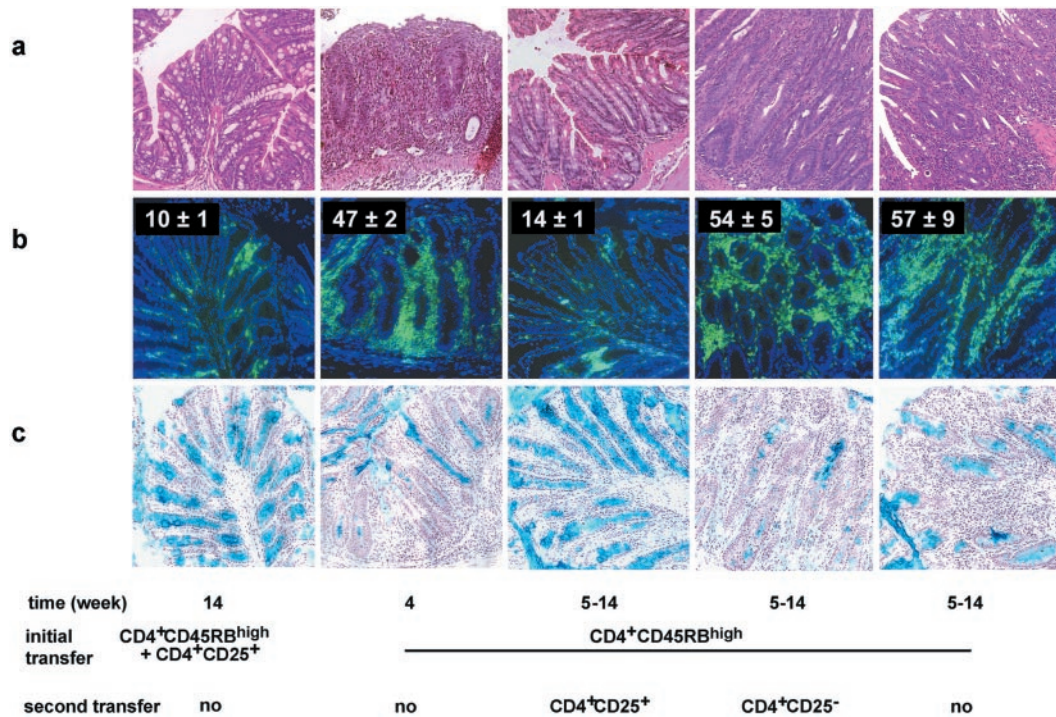


FIGURE 2. Histological sections of intermediate to distal colon. Initial cotransfer of CD4⁺CD45RB^{high} and CD4⁺CD25⁺ T cells into *rag1*^{-/-} mice prevented the development of colitis as illustrated by normal histology. In contrast, control mice developed moderate to severe colitis after CD4⁺CD45RB^{high} T cell transfer. Transfer of CD4⁺CD25⁺ but not of CD4⁺CD25⁻ T cells into colitic mice ameliorates the colitis. *a*, H&E staining. *b*, CD4 staining and DAPI counterstaining. *Insets* show the CD4⁺ cell density in cells per 10,000 μm² (average of at least four mice per group ± SEM). *c*, Alcian blue and periodic acid-Schiff staining to visualize goblet cells and mucus deposition. Microphotographs were taken at ×200 magnification.

CD4⁺CD25⁺ T cells home to the MLN and colon

The difference in the ability of CD4⁺CD25⁺ and CD4⁺CD25⁻ populations to resolve intestinal inflammation may reflect differences in their homing or survival *in vivo*. To examine the distribution of these cells *in vivo*, colitis was induced in *rag1*^{-/-} mice by transfer of CD4⁺CD45RB^{high} T cells from CD45.2⁺ mice. Colitic mice were then injected with CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells from congenic CD45.1⁺ donors allowing detection of the progeny using FACS and immunofluorescence.

Two to 3 wk after transfer of CD4⁺CD25⁺ T cells, all mice still had marked inflammation in the colon. The frequency of the progeny of CD4⁺CD25⁺ T cells was low in MLN, spleen, and LP (0.7–4.8% of total CD4⁺ T cells; Fig. 3*a*). By 10 wk posttransfer, the mean frequency increased significantly to 30.2% in the spleen, 40.7% in the MLN, and 17.7% in the LP (Fig. 3*a*). This increase in frequency was mirrored by an increase in the absolute numbers of CD4⁺CD25⁺ progeny in spleen (from 7 ± 5 to 33 ± 18 × 10⁴; NS), MLN (from 2 ± 1 to 32 ± 7 × 10⁴; *p* = 0.016), and LP (from 11 ± 3 to 67 ±

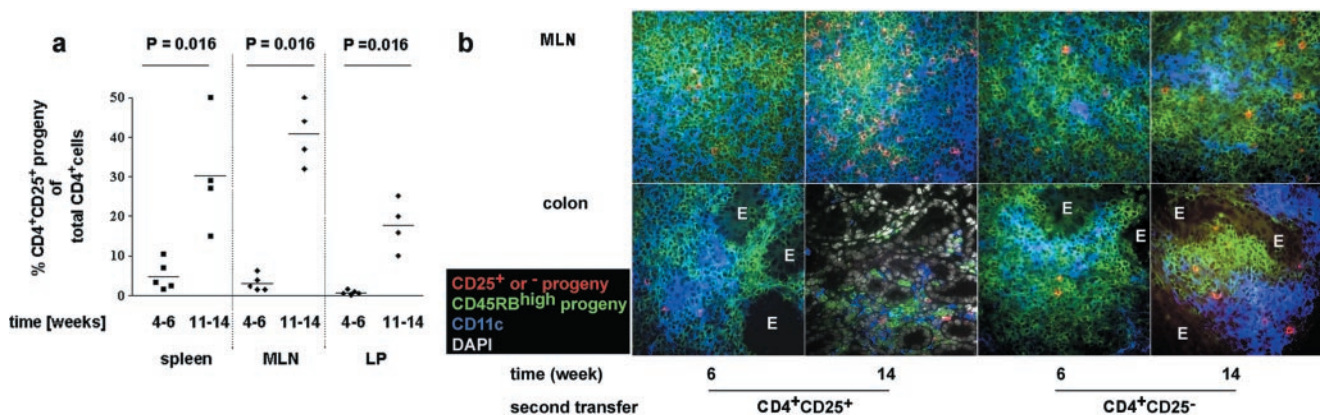


FIGURE 3. Distribution of CD4⁺CD25⁺ T cells after transfer into *rag1*^{-/-} colitic mice. *a*, Percentage of CD4⁺CD25⁺ T cell progeny among total CD4⁺ T cell in spleen, MLN, and LP after transfer of congenic CD4⁺CD25⁺ cells. *b*, Immunostaining for CD4⁺CD45RB^{high}, CD4⁺CD25⁺, or CD4⁺CD25⁻ cell progeny as well as CD11c⁺ cells. CD4⁺CD25⁺ and CD4⁺CD25⁻ cells are distributed similarly in MLN and colon in the first 2 wk after transfer (wk 6). However, the CD4⁺CD25⁺ progeny accumulates in the MLN and reverses colitis as indicated by reduction of the CD4⁺ and CD11c⁺ cell infiltrate. To illustrate the reduction of the LP infiltrate in CD4⁺CD25⁺-injected mice at the end of the experiment, the DAPI nucleus staining is shown to identify multiple epithelial cell areas (E). Microphotographs were taken at ×400 magnification.

36×10^4 ; NS). During the first 2 wk, four of five of the CD4⁺CD25⁻-injected mice had to be sacrificed. The frequency of CD4⁺CD25⁻ progeny was low (<2% in spleen, MLN, and LP). Interestingly, although inconclusive, in the surviving mouse, the frequency remained low up to 10 wk after transfer (6% in MLN and <2% in spleen and LP). Histological analysis of MLN and colon sections confirmed the FACS data with a low but similar density of both CD4⁺CD25⁺ and CD4⁺CD25⁻ T cell progeny 2 wk after their transfer and an increased density of CD4⁺CD25⁺ but not CD4⁺CD25⁻ T cell progeny in the MLN and LP sections at 10 wk posttransfer (Fig. 3*b*).

The progeny of CD4⁺CD25⁺ T cells proliferate in MLN and colon

To examine the influence of CD4⁺CD25⁺ T cells on local T cell proliferation and to identify where CD4⁺CD25⁺ progeny proliferate in vivo, we examined the histological expression of the proliferation marker Ki67, which is specifically expressed and tightly regulated during cell proliferation (13) (Fig. 4*a*). In colitic mice, CD4⁺CD45RB^{high} progeny were found to be proliferating at wk 4 and 14 in both the MLN (35 vs 29%) and inflamed LP (17 vs 19%) (Fig. 4*b*). Two weeks after injection of CD4⁺CD25⁺ T cells, the frequency of Ki67 expression among CD45RB^{high} progeny was similar to that of mice that did not receive T_R cells. At this time point, a significant proportion of CD4⁺CD25⁺ T cell progeny also showed Ki67 expression in MLN (30 ± 6%) and LP (33 ± 6%), indicating an active expansion of this cell population in both compartments (Fig. 4*b*). In contrast, 10 wk after transfer of CD4⁺CD25⁺ T cells, when the inflammation in the colon had resolved, the frequency of proliferating cells among the progeny of both CD4⁺CD45RB^{high} as well as CD4⁺CD25⁺ T cells was significantly reduced in MLN and LP (Fig. 4*b*). Taken together, these data indicate that early after transfer into colitic mice, CD4⁺CD25⁺ T_R cells proliferate in MLN and colon and that resolution of the inflammatory response correlates with a substantially reduced number of proliferating pathogenic cells.

The finding that CD4⁺CD25⁺ T_R cells expand in the spleen and the MLN after transfer into immunodeficient mice is consistent with a previous report (14). However, our results show in addition that, under inflammatory conditions, not only clas-

sical effector T cells but also T_R cells accumulate and proliferate in the intestinal mucosa. These results raise the possibility that T_R cells control effector T cell responses not only in the lymph node but also in the inflamed tissue. Similar results have been described in a model of transplantation tolerance (15). Induction of T_R cell proliferation in vitro via enhanced costimulation coincides with the loss of their suppressor function (8, 9). After expansion in vivo, CD4⁺CD25⁺ T_R cells were found to be more potent suppressors in vitro (16). Our data also suggest that, in vivo, under inflammatory conditions, vigorous proliferation does not lead to a loss of suppressor function as assessed by resolution of inflammation.

CD4⁺CD25⁺ T cells are in contact with CD11c⁺ cells and CD4⁺ T cells in vivo

To determine the localization of CD4⁺CD25⁺ T cells in relation to CD11c⁺ cells and the progeny of CD4⁺CD45RB^{high} T cells, we analyzed the histological distribution of these cells in MLN and colon LP. The CD4⁺CD25⁺ T cell progeny were found to be in direct contact with the CD4⁺CD45RB^{high} progeny as well as, in >90% of cases, with CD11c⁺ cells (Fig. 3*b*), predominantly located between clusters of CD11c⁺ cells and CD4⁺CD45RB^{high} T cell progeny. In the colon, this localization pattern of the T_R cells was seen 2 wk posttransfer in the presence of the inflammatory infiltrate as well as in the remaining leukocytic clusters in the LP 10 wk posttransfer, indicating that direct physical contact of CD4⁺CD25⁺ cells with CD11c⁺ cells was a consistent pattern.

Previous studies have shown that interactions between pathogenic T cells and CD11c⁺ cells within MLN and colon are important for the initiation of colitis in this model (17, 18). The observed location of T_R cells at the interface of APC and effector T cells supports a role for APC-T_R interactions in T_R function, i.e., in T_R cell activation and/or migration (19). In addition, T_R cells may regulate the activation state of the APC itself, which might thus interfere with their ability to activate effector T cell responses (20). Furthermore, the ability of T_R cells to resolve established colitis may also involve direct T_R-T effector cells interactions (7–9).

In summary, our data show that T_R cell activity in vivo has the potential to reverse established inflammation leading to cure of colitis. Cell therapy with regulatory cells has some clear

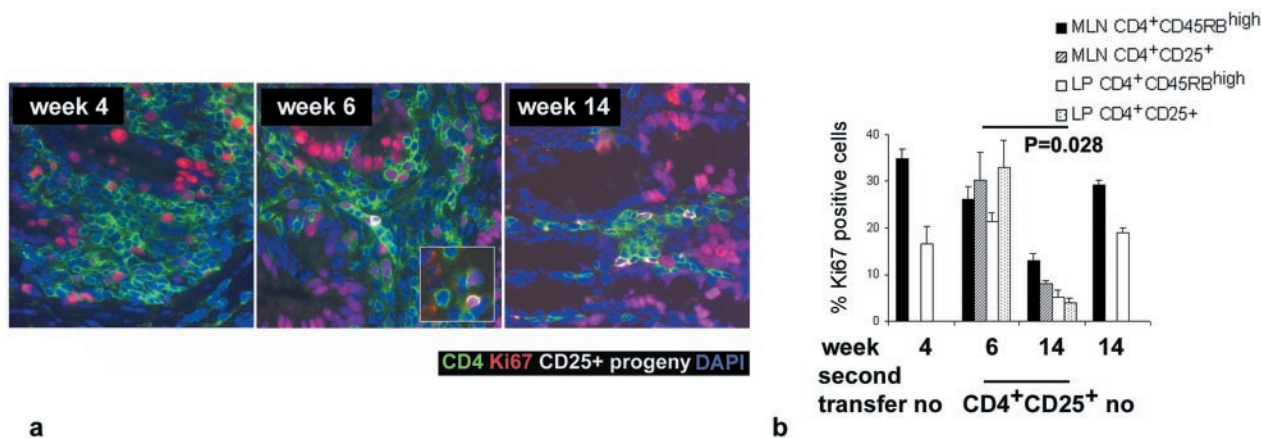


FIGURE 4. CD4⁺CD25⁺ T cells proliferate in MLN and LP after transfer into *rag1*^{-/-} colitic mice. *a*, Ki67 expression in the colon LP. Staining for Ki67, CD4, CD45.1, and DAPI is shown for mice without second T cell transfer (wk 4), as well as early (wk 6) and late (wk 14) after CD4⁺CD25⁺ T cell transfer. *Inset* shows Ki67-positive cell of the CD4⁺CD25⁺ progeny 2 wk after CD4⁺CD25⁺ transfer. Microphotographs were taken at ×630 magnification. *b*, Percentage of Ki67-positive in the progeny of CD4⁺CD45RB^{high} or CD4⁺CD25⁺ T cells in MLN and colon LP. Mean and SEM are shown for *n* ≥ 4 mice.

advantages. These include their ability to migrate to inflammatory sites and to influence Th1 and Th2 responses (21) as well as a potential for homeostatic and self-limited expansion.

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