Foxp3-Expressing CD103⁺ Regulatory T Cells Accumulate in Dendritic Cell Aggregates of the Colonic Mucosa in Murine Transfer Colitis

Frank Leithäuser,* Tamara Meinhardt-Krajina,^{†‡}
Kerstin Fink,[†] Beate Wotschke,* Peter Möller,*
and Jörg Reimann[‡]

From the Departments of Pathology* and Medical Microbiology and Immunology,[‡] University of Ulm, Ulm; and the Department of Medical Microbiology and Hygiene,[†] University of Tübingen, Tübingen, Germany

Little is known of the anatomical compartmentalization of colitogenic or regulatory T-cell responses in the murine transfer colitis model. Therefore, we analyzed the putative function of large intestinal dendritic cell (DC) aggregates, to which donor CD4⁺ T cells selectively home before colitis becomes manifest. The co-stimulatory molecules MHC-II, CD40, CD80, and CD86 were expressed in DC aggregates. IL-23 was primarily absent from DC aggregates at all stages of disease but was expressed at high levels in the severely inflamed lamina propria. Interferon- γ was up-regulated in the lamina propria during early and advanced disease, whereas in DC aggregates it was detectable to a significant degree only in fully developed colitis. In contrast, Foxp3, a marker of regulatory T cells, was expressed in DC aggregates on T-cell transfer, coinciding with the appearance of CD103⁺ CD25⁻ T cells in these clusters. Foxp3 was enriched in the CD103⁺ T-cell fraction isolated from the lamina propria of diseased mice. T-cell grafts depleted of CD103⁺ T cells generated similar numbers of colonic CD103⁺ T cells as unfractionated T cells. We conclude that DC aggregates are structures involved in the expansion and/or differentiation of CD103⁺ CD25⁻ CD4⁺ Foxp3-expressing regulatory T cells. (*Am J Pathol* 2006, 168:1898–1909; DOI: 10.2353/ajpath.2006.050228)

The normal intestinal immune system responds to pathogenic but not commensal bacteria colonizing the gut. This tightly balanced system occasionally fails, leading to local immunopathology. Crohn's disease and ulcerative

colitis, the two most common inflammatory bowel diseases (IBDs) of man, may result from a deregulated immune reaction to the intestinal microflora.¹ Specifically, an aberrant T-cell response is thought to play a pivotal role in the pathogenesis of IBD. Yet, how T-cell reactions lead to intestinal inflammation is unresolved.

Animal models have been used to elucidate the pathogenesis of IBD.² In the transfer colitis model, the reconstitution of immunodeficient mice with naive, immunocompetent CD4⁺ T cells from congenic donors induces a severe colitis that mimics some aspects of human IBD. In this model the naive CD45RB^{high} T-cell graft undergoes activation and Th1 polarization and becomes colitogenic.³ Co-transfer of CD45RB^{low} CD4⁺ T cells can antagonize the disease-inducing capacity of the CD45RB^{high} T-cell fraction and prevent the manifestation of IBD.^{4,5} There is abundant evidence that IBD is also inducible by transfer of unfractionated, mature CD4⁺ T cells (containing a large population of CD45RB^{high} T cells and a small subset of CD45RB^{low} CD4⁺ T cells).⁶ As in most experimental IBD systems, the intestinal bacterial flora is required for disease manifestation in the transfer colitis model. This is supported by the finding that animals kept under germ-free conditions fail to develop IBD.⁷

Intestinal immune responses take place within a complex system of (micro)anatomical compartments,⁸ usually subdivided into the lamina propria, the local and regional lymph nodes, and the gut-associated lymphatic tissue.⁹ The latter comprises Peyer's patches and intestinal lymphoid aggregates (ILAs) located within the small and large intestinal wall in close proximity to the epithelial lining.¹⁰ These small structures of the murine gut have been neglected for a long time but have recently attracted attention. Their detailed analysis led to the dis-

Supported by the Deutsche Forschungsgemeinschaft (grant DFG LE 1331/3-1 to F.L.) and the University of Ulm (Interdisziplinäres Zentrum für Klinische Forschung E6 to P.M. and F.L.).

Accepted for publication February 14, 2006.

Address reprint requests to Frank Leithäuser, Department of Pathology, University of Ulm, Albert Einstein Allee 11, 89081 Ulm, Germany. E-mail: frank.leithaeuser@medizin.uni-ulm.de.

crimination between cryptopatches and isolated lymphoid follicles (ILFs), two apparently distinct entities. Cryptopatches were defined as tiny aggregates of c-kit⁺ IL-7R⁺ cells randomly scattered throughout the intestinal mucosa that lack mature lymphocytes.¹¹ ILFs were characterized as solitary B-cell follicles that are localized in the anti-mesenteric region of the intestinal wall and contain small numbers of mature T lymphocytes but also c-kit⁺ and IL-7R⁺ cells.^{12,13} However, the classification of ILAs into either cryptopatches or ILFs was recently challenged.¹⁰ Analyzing a large number of such aggregates, it was shown that most of them display properties intermediate between cryptopatches and ILFs.

To date, the function of ILAs is not fully understood. ILAs have been analyzed mainly under steady-state conditions in the absence of disease. Furthermore, there is little information on the anatomical compartmentalization of different T-cell functions involved in the pathogenesis of IBD. We previously reported that donor T cells accumulate and proliferate in conspicuous, subepithelial dendritic cell (DC) aggregates located in regions of the colonic lamina propria where inflammation later develops.¹⁴ T-cell clustering is obvious early in IBD development before overt colitis is established. This prompted us to hypothesize that Th1 cell priming occurs in DC aggregates. In the present study, we demonstrate that these DC structures represent the equivalent to ILAs and address in detail their functional properties in the context of IBD.

Materials and Methods

Mice

C57BL/6J (B6) mice, C57BL/6J-*Rag1*^{tm1Mom} (RAG^{-/-}) B6 mice¹⁵ (stock no. 2216; Jackson Laboratories, Bar Harbor, ME), TCR transgenic OT-II B6 mice¹⁶ (stock no. 004194, Jackson Laboratories), and eGFP-tg B6 mice¹⁷ (kindly provided by Dr. M. Okabe, Osaka, Japan) were used. Mice were kept under specific pathogen-free conditions in the animal facility of Ulm University and transplanted at 8 to 12 weeks of age. All experiments were approved by our institutional animal care committee.

Isolation and Adoptive Transfer of GFP⁺ CD4⁺ Cells

CD4⁺ T cells were purified from spleen cells of normal B6, eGFP-tg B6, and OT-II B6 mice using the CD4 isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) as described.¹⁸ eGFP-tg B6 mice express eGFP under the chicken β -actin promoter control.¹⁷ eGFP is heterogeneously expressed from the transgene in the CD4⁺ T-cell population of transgenic mice but remains stable within the adoptive host.¹⁹ The purity of isolated CD4⁺ T cells was >95 to 98% on reanalysis. CD4⁺ T cells (3×10^5) were injected intraperitoneally.

Tissue Processing

Nontransplanted RAG^{-/-} mice and RAG^{-/-} mice at 2 weeks and 4 weeks after adoptive CD4⁺ T-cell transfer were analyzed. Animals were euthanized, and the entire small and large intestine was removed. For the detection of eGFP⁺ cells,¹⁹ tissue was fixed in 4% buffered paraformaldehyde solution, snap-frozen in liquid nitrogen, and stored at -70°C. For immunohistological staining as well as for microdissection and subsequent RNA isolation, tissue was immediately snap-frozen in liquid nitrogen and stored at -70°C.

Immunohistology and Detection of eGFP⁺ Cells

All immunohistological stainings were performed at least twice on two separate mice using longitudinal sections of the entire large intestine. Monoclonal antibodies (mAbs) used for immunohistology were as follows: CD3 ϵ clone 500A2, CD11c clone HL3, anti-c-kit clone 2B8, CD40 clone 3/23, CD80 clone 16-10A1, CD86 clone GL1, CD103 clone M290, anti-MHC-II clone 2G9 (all mAbs from BD Biosciences, Heidelberg, Germany), CD127 (IL-7R) clone A7R34 (eBioscience, San Diego, CA), CD25 clone PC61 5.3 (Caltag, Hamburg, Germany). Anti-laminin (DPC Bierman, Bad Nauheim, Germany) was a polyclonal rabbit antibody. Antibodies against CD11c, CD25, CD40, CD80, CD86, and CD103 were biotin-labeled, all other antibodies were unconjugated. Single immunohistological staining was performed with a peroxidase-conjugated donkey anti-rat IgG (Dianova, Hamburg, Germany) to detect MHC-II. Bound CD40, CD80, and CD86 were detected by signal amplification using the biotin-XX kit (Molecular Probes, Göttingen, Germany) following the instructions of the manufacturer. For double staining of CD3 and CD11c, Cy2-conjugated goat anti-Syrian hamster IgG (Dianova) was used to detect bound CD3, and Cy3-conjugated goat anti-Armenian hamster IgG (Dianova) was used to detect bound CD11c. For double staining of c-kit and CD11c, the same reagent was used to detect bound CD11c and biotin-conjugated mouse anti-rat IgG (Dianova) followed by signal amplification with the Alexa Fluor-488 kit (Molecular Probes) revealed bound anti-c-kit. For double staining of CD127 and CD11c, the same reagent was used to detect bound CD11c, and Alexa Fluor-488-conjugated donkey anti-rat IgG (Molecular Probes) revealed bound CD127. For double staining of CD3 and CD25, Cy3-conjugated goat anti-Syrian hamster IgG (Dianova) was used to detect bound CD3, and bound CD25 was revealed by signal amplification using the Alexa Fluor-488 kit (Molecular Probes). Double labeling of CD3 and CD103 was performed using the same reagent to detect bound CD3 and Alexa Fluor-488-conjugated donkey anti-rat IgG (Molecular Probes) to reveal bound CD103. Double staining of CD11c and laminin was performed with Cy3-conjugated goat anti-Armenian hamster IgG (Dianova) to detect bound CD11c, and Cy2-conjugated goat anti-rabbit IgG (Dianova) served to reveal anti-laminin antibody.

Acetone-fixed frozen tissue sections were incubated with the primary antibodies at predetermined dilutions for

1 hour, washed twice in phosphate-buffered saline (PBS), and incubated with the appropriate secondary antibodies or streptavidin, respectively. In negative control stainings of murine antigens, isotype-matched mAbs to an irrelevant target were used. All incubation steps were performed at room temperature in PBS.

Cryosections from paraformaldehyde-fixed tissue (to detect eGFP⁺ cells) and immunofluorescent stainings were counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich, Deisenhofen, Germany) and embedded in Cytoseal medium (Microm, Walldorf, Germany). Slides were examined under an Axioskop microscope (Zeiss, Jena, Germany). Pictures were recorded by a charge-coupled device camera and processed on a computer using the Photoshop software (version 5.5; Adobe, Unterschleißheim, Germany). For immunohistochemical staining, bound peroxidase activity was visualized by the substrate 3-amino-9-ethylcarbazole. Sections were counterstained in hematoxylin.

In Situ Staining of Foxp3

Two- μ m sections were cut from formalin-fixed, paraffin-embedded tissue and subjected to an antigen retrieval treatment performed in 10 mmol/L citrate buffer (pH 6.0) in a pressure cooker. Next, sections were incubated with the rat anti-Foxp3 mAb clone FJK-16s (eBioscience), followed by peroxidase-conjugated donkey anti-rat IgG (Dianova). The subsequent color substrate reaction and counterstaining was as described above.

Assessment of Colitis Intensity

A semiquantitative assessment of disease intensity was performed on conventional hematoxylin and eosin (H&E)-stained slides applying a three-step histological score as described elsewhere.¹⁸

Microdissection and Laser Pressure Catapulting

Membrane-covered glass slides were mounted with 7- μ m frozen tissue sections, stained with hematoxylin, dried, and stored at -70°C . Microdissection and laser pressure catapulting was performed using a Robot-Microbeam system (P.A.L.M. Microlaser Technologies, Bernried, Germany) equipped with an IX50 microscope (Olympus, Hamburg, Germany). Several tissue areas comprising a minimum of 1000 cells were catapulted into the cap of one polymerase chain reaction (PCR) tube. Lamina propria tissue was isolated after excising crypts, including intraepithelial lymphocytes. Contamination of the lamina propria with these components could not be excluded but was likely to be low.

RNA Isolation and Reverse Transcription

RNA was isolated from microdissected tissue using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA) following the manufacturer's instructions, including a

DNase treatment. Eluted RNA was stored at -70°C . To prepare RNA from lamina propria T cells, cells were lysed in Trizol reagent (Life Technologies, Karlsruhe, Germany) and RNA was extracted according to the manufacturer's instructions. Twelve μ l of RNA isolated from microdissected tissue or 1 μ l of RNA prepared from cell suspensions, respectively, were annealed to 1 pmol of hexamer (random) primer, denatured at 80°C and reverse-transcribed as described.¹⁸

Real-Time PCR

mRNA expression was determined by real-time reverse transcriptase (RT)-PCR and relative quantification using the reference gene HPRT or CD3, respectively. Primers for HPRT and Foxp3 were as published elsewhere.²⁰ For all other targets, intron-spanning primers were designed to minimize amplification of traces of DNA. Primer sequences were: CD3 forward 5'-ATATCTCATTGCGGGA-CAGG-3', CD3 reverse 5'-TCCTCAGTTGGTTTCCTTGG-3', IL-23p19 forward 5'-AATAATGTGCCCGTATCCA-3', IL-23p19 reverse 5'-AGGCTCCCCTTTGAAGATGT-3', interferon (IFN)- γ forward 5'-GCTTTCAGCTCT-TCCTCAT-3', IFN- γ reverse 5'-GTCACCATCCTTTGC-CAGT-3', transforming growth factor (TGF)- β 1 forward 5'-TGCGCTTGCAGAGATTAAAA-3', TGF- β 1 reverse 5'-CGTCAAAAGACAGCCACTCA-3'. PCR was performed in the iCycler iQ Real-Time PCR instrument (Bio-Rad, München, Germany). The regression coefficient of standard curves was always between -0.98 and -1.00 . Quantification was calculated according to an established mathematical model.²¹ All PCR runs were performed at least twice using two separate mice.

Preparation of Colonic Lamina Propria Cells, Flow Cytometry Analysis, and Cell Sorting

Intestines were washed in PBS, and the gut epithelium was removed from the lamina propria as described.¹⁸ The tissue was digested with 0.5 mg/ml of collagenase type VIII and 5 U/ml DNase at 37°C for 90 minutes in RPMI 1640/5% v/v fetal calf serum. Lamina propria lymphoid cells were collected at the 40%/70% interface of a Percoll gradient for analysis by flow cytometry or fluorescence-activated cell sorting.

Four-color flow cytometry analysis was performed on a FACScalibur (Becton-Dickinson, Mountain View, CA) device. The following mAbs were used: fluorescein isothiocyanate- or PerCP-conjugated CD3 ϵ clone 145-2C11, phycoerythrin-conjugated CD103 clone M290, APC-conjugated CD4 clone RM4-5 (mAbs from BD Biosciences) for surface staining and fluorescein isothiocyanate anti-mouse Foxp3 clone FJK-16s (eBioscience) for intracellular staining, following the manufacturer's instructions. CD4⁺ T-cell subsets were sorted on a FACStar Plus (Becton-Dickinson) cell sorter. Purity of sorted populations was always $\geq 99.5\%$ on reanalysis.

Figure 1. Accumulation of donor T cells in DC aggregates of the large intestine that contain c-kit⁺ and IL-7R⁺ cells. RAG^{-/-} were reconstituted with CD4⁺ T cells and analyzed 2 or 4 weeks after transplantation. At 2 weeks (A), H&E staining of the large intestine revealed mild inflammatory changes, whereas severe colitis was manifest 4 weeks after reconstitution (B). C: Histological colitis scores are depicted as the mean and SD of five mice per group. The *P* value is given as determined by Student's *t*-test. D and E: Genetically tagged CD4⁺ T cells from eGFP-transgenic donor mice were used to track their colonization of the intestinal mucosa in the adoptive host. D: Two weeks after transplantation, T cells selectively accumulated in conspicuous clusters in the colonic mucosa. Four weeks after T-cell transfer, a dense and diffuse infiltration of the lamina propria by eGFP⁺ T cells was seen (E) but T-cell clustering was not prominent. Double staining of CD3 (green) and CD11c (red) of intestinal tissue at 2 weeks after T-cell transfer (F, G) confirmed T-cell accumulation in CD11c⁺ DC aggregations in the large intestine (F) while few T cells clustered in the small intestine (G). CD11c⁺ (red) DC aggregates of untransplanted RAG^{-/-} mice harbored numerous c-kit⁺ cells (green, H) and IL-7R⁺ cells (green, I). Original magnifications: $\times 10$ (A, B, D–G); $\times 20$ (H, I).

Suppression Assay

Naive CD4⁺ T cells were isolated from OT-II B6 mice. Splenic DCs (5×10^4) from normal B6 mice were pulsed for 2 hours with the A^b-binding OVA_{323–339} peptide ISQAVHAAHAEINEAGR (recognized by the transgene-encoded TCR expressed by OT-II B6 mice), washed, and co-cultured with 2.5×10^5 CD4⁺ OT-II T cells in 200- μ l flat-bottom wells in RPMI 1640 medium with 5% fetal calf serum. Sorted colonic lamina propria CD4⁺ CD103⁺ or CD4⁺ CD103⁻ T cells were added in co-culture (5×10^4 cells/well). After 72 hours the supernatants were collected, and their IFN- γ production was measured by double-sandwich enzyme-linked immunosorbent assay.

Statistical Analysis

Data were analyzed for statistical significance using the Student's *t*-test.

Results

T Cells Cluster in ILAs

Two weeks after adoptive T-cell transfer, a mild colitis was observed by histopathology showing a loose inflammatory infiltrate, stromal edema, and reduction of goblet cell number in the cryptal epithelium (Figure 1A). Four weeks after reconstitution, the large intestine displayed the histological alterations of severe colitis, characterized by a dense inflammatory infiltrate, loss of goblet cells, and a distortion of the mucosal architecture (Figure 1B). The different IBD intensities in both groups were confirmed by means of a semiquantitative colitis score (Figure 1C). In agreement with our earlier observations,¹⁴ genetically labeled eGFP⁺ donor T cells accumulated in focal aggregates located in the intestinal lamina propria. This clustering was apparent in the initial phase of disease development at day 14 after transfer (Figure 1D), before the manifestation of overt IBD. In areas of severe colitis at 28 days after

Figure 2. Intraepithelial DCs are associated with DC aggregates and display a mature phenotype. **A** and **B**: Double staining of CD11c (red) to label DCs and laminin (green) to visualize the basal membrane. Intraepithelial CD11c⁺ DCs (red) located at the luminal side of the basal membrane are predominantly found in association with DC aggregates (arrowheads in **B**). Note a DC passing a gap in the basal membrane (arrow in **B**). Single immunohistochemical staining of MHC-II (**C**) reveals predominant expression of MHC-II (**C**), CD40 (**D**), CD80 (**E**), and CD86 (**F**) in DC aggregates. Original magnifications: $\times 10$ (**A**, **C–F**), $\times 40$ (**B**).

T-cell transfer, T-cell clustering was not evident (Figure 1E). However, when the entire length of the large intestine was examined by histology, T-cell clusters were identified even at this late time point in areas with less severe inflammation. Double immunostaining of CD3 and CD11c demonstrated that T cells co-localized with

DCs (Figure 1F). DC aggregates displayed a considerable variation in size that apparently did not have any influence on T-cell accumulation. T lymphocytes were also found in DC aggregates of the small intestine but were generally lower in number than in the large bowel (Figure 1G).

To elucidate the nature of DC aggregates, we determined their expression of c-kit and the IL-7 receptor (IL-7R). We found c-kit⁺ and IL-7R⁺ cells in DC aggregates (Figure 1, H and I). Based on their similarity in morphology and surface marker expression, we conclude that these DC aggregates found in RAG^{-/-} mice represent the equivalents of ILAs¹⁰ of immunocompetent mice which comprise intestinal lymphoid follicles, cryptopatches, and lymphoid structures with intermediate properties.

DCs in Aggregates Are Closely Associated with the Epithelium and Display a Mature Phenotype

DCs have been shown to colonize the follicle-associated epithelium of Peyer's patches.²² Using double staining for CD11c and laminin (a collagen constituent of the basal membrane) we observed numerous CD11c⁺ DCs at the luminal site of the basal membrane within the epithelium. Intraepithelial DCs were found predominantly in the epithelium overlying the DC aggregates and only rarely detected in the epithelium outside these organized lymphoid structures (Figure 2A). Some CD11c⁺ cells were observed crossing the basal membrane (Figure 2B) suggesting a high turnover rate of DCs within the intraepithelial compartment. The outcome of DC/CD4⁺ T-cell interactions critically depends on the maturation stage of the DCs.²³ Surface markers that indicate a mature DC phenotype are MHC-II, CD40, CD80, and CD86. We determined the expression of these molecules in the large intestinal mucosa by immunohistology. As previously described, MHC-II is highly expressed in DC clusters of RAG^{-/-} mice,¹⁴ now identified to represent ILAs (Figure 2C). A faint CD40 staining was detectable in a subset of DCs in the aggregates but not in the lamina propria (Figure 2D). Strong expression of CD80 was found in the vast majority of cells in DC aggregates (Figure 2E) but only occasionally seen in the lamina propria. CD86 staining was detectable in DC aggregates and in the lamina propria (Figure 2F).

Distinct Cytokine Expression Pattern by DCs in Aggregates and in the Lamina Propria

To gain insight into the functional properties prevalent in ILAs and the lamina propria, we determined the mRNA expression of cytokines indicative of Th1 polarization. Tissue from DC aggregates and lamina propria (depleted from cryptal epithelium) isolated by microdissection and laser capture catapulting (Figure 3A) was submitted to quantitative RT-PCR. This was performed using material from untransplanted RAG^{-/-} mice and RAG^{-/-} mice at day 14 and day 28 after CD4⁺ T-cell reconstitution. Before transplantation and 2 weeks after adoptive transfer, the expression level of IL-23 p19 mRNA (Figure 3B) was low in DC aggregates and virtually undetectable in the lamina propria. At day 28, expression was markedly enhanced in the lamina propria but remained low in DC aggregates. IFN- γ expression, normalized against the

Figure 3. IL-23p19 and IFN- γ expression in microcompartments of the large intestine. Tissue from the lamina propria (**A, top row**) and DC aggregates (**A, bottom row**) was obtained by microdissection and subjected to quantitative RT-PCR. The process of microdissection is illustrated in **A**, showing histological sections stained with hematoxylin (**left**), after laser microdissection and removal of the epithelial crypts (**center**), and after catapulting the dissected tissue compartments (**right**). Epithelia (including intraepithelial lymphocytes) were carefully excised to minimize contamination of microdissected lamina propria with these components. The mRNA expression of IL-23p19 (**B**) and IFN- γ (**C**) was determined in the lamina propria (**light columns**) and DC aggregates (**dark columns**) in untransplanted RAG^{-/-} mice (day 0), at day 14 and day 28 after CD4⁺ T-cell transfer. The mean mRNA expression (of three samples) and the SD were calculated after normalization to HPRT (**B**) and CD3 (**C**), respectively, and are shown in arbitrary units. *P* values are given as determined by Student's *t*-test. Asterisks indicate no detectable mRNA expression. Original magnifications, $\times 10$.

T-cell density by using CD3 as a housekeeping gene, was already strongly induced in the lamina propria at day 14, when it was hardly found in DC aggregates (Figure 3C). Four weeks after transfer (when the IBD was severe), IFN- γ maintained essentially the same expression level in the lamina propria, whereas it was up-regulated in ILFs.

Foxp3-Expressing Regulatory T Cells Reside in DC Aggregates of Transplanted RAG^{-/-} Mice

Because the analysis of the cytokine expression provided no evidence for a Th1-polarization in DC aggregates, we tested if regulatory T cells are present in these organized lymphoid structures. We focused on the T_{reg} cell population, which has been shown to play a prominent role in controlling transfer colitis.²⁴ This subset is included in the CD45RB^{low} CD4⁺ T-cell fraction that inhibits IBD induction by CD45RB^{high} donor T cells. T_{reg} cells are characterized by the expression of the transcription factor

Figure 4. Fcpx3 expression in the large intestine. The mRNA expression (A) of Fcpx3 was quantified applying CD3 as a housekeeping gene as explained in the legend to Figure 3 and is shown as the mean and SD of three measurements. *P* values are given as determined by Student's *t*-test. Asterisks indicate no detectable mRNA expression. Light columns indicate the lamina propria; dark columns indicate DC aggregates. B–E: Immunohistochemistry with a monoclonal Fcpx3 antibody reveals nuclear labeling of cells in the medullary thymus (B, right) but not in the cortex (B, left), proving the specificity of the staining. In the large intestine of mice at day 14 after transplantation (C, D), several Fcpx3-expressing cells are found in DC aggregates, whereas few labeled cells are seen in the lamina propria. E: At day 28 after transplantation, Fcpx3⁺ cells are present at low density in the severely inflamed gut. Arrow in E indicates a crypt abscess. Original magnifications: ×20 (B, C, E); ×40 (D).

Fcpx3, the only known molecular marker for this regulatory T-cell subset.²⁰ We first measured the expression of Fcpx3 by quantitative RT-PCR using CD3 as a housekeeper. As expected for a T-cell-deficient mouse, Fcpx3 was undetectable in untransplanted RAG^{-/-} mice (Figure 4A). Two weeks after T-cell transfer, substantial levels of Fcpx3 mRNA were found in DC aggregates. In the lamina propria, Fcpx3 expression was lower than in DC aggregates, although this difference was not quite significant (*P* = 0.0842). At day 28, Fcpx3 was markedly down-regulated in DC aggregates and in the lamina propria. We then determined Fcpx3 expression *in situ* by immunohistochemistry on formalin-fixed, paraffin-embedded tissue. This staining detected Fcpx3⁺ cells in the thymic medulla of normal B6 mice (Figure 4B) at a similar pattern and density as has been recently reported in mice harboring a GFP-Fcpx3 fusion protein-reporter knockin allele,²⁵ demonstrating the specificity of the immunohistochemistry. In line with the mRNA findings, nu-

merous Fcpx3⁺ cells were observed in DC aggregates of mice with early disease, but few cells producing this antigen could be seen in the lamina propria (Figure 4, C and D). At advanced IBD, Fcpx3-expressing cells colonized the lamina propria at a low density (Figure 4E).

CD25⁺ CD103⁺ T Cells Are Present in DC Aggregates

The most widely used surface molecule to identify T_{reg} cells is the IL-2 receptor α -chain (CD25).²⁴ We therefore performed double-immunohistological staining to determine whether CD25⁺ T_{reg} cells are present in DC aggregates. T-cell-reconstituted mice at day 14 after transfer harbored CD25⁺ cells in DC aggregates. However, these cells did not co-express CD3 (Figure 5, A and B), and therefore do not represent T_{reg} cells. CD25⁺ cells in DC aggregates are unlikely to be of donor origin because these cells were also abundant in DC aggregates of untransplanted RAG^{-/-} mice (Figure 5C). In contrast to CD25, a substantial fraction of CD3⁺ T cells in DC aggregates expressed CD103 (Figure 5, D and E), a surface molecule defining a recently described adaptive T_{reg} cell subset.^{26–28} CD103⁺ T cells were also identified in the lamina propria outside of ILFs (Figure 5F) and tended to display a stronger CD103 expression than T cells that localized to DC aggregates. To confirm that the CD103⁺ expression was associated with a T_{reg} cell phenotype, we electronically sorted CD103⁺ and CD103⁻ T-cell fractions from the lamina propria (including DC aggregates) of T-cell-reconstituted mice (Figure 5G). As shown in Figure 5H, high levels of Fcpx3 mRNA expression were detected only in the CD103⁺ fraction.

TGF- β 1 mRNA Is Expressed in DC Aggregates

TGF- β has been implicated in the extrathymic differentiation of naive CD4⁺ T cells into T_{reg} cells.²⁹ Moreover, TGF- β can induce CD103.³⁰ We therefore measured TGF- β 1 mRNA expression in DC aggregates and in the lamina propria of the large intestine. We detected high TGF- β 1 mRNA expression in DC aggregates but not in the lamina propria of untransplanted RAG^{-/-} mice (Figure 6A). At 2 weeks and 4 weeks after T-cell transfer, there was no significant change in the amount of TGF- β 1 message in DC aggregates. In contrast, TGF- β 1 expression increased steadily in the colonic lamina propria to reach levels comparable to those in DC aggregates at day 28 after transfer. It is unlikely that CD4⁺ T cells are the source of TGF- β 1 mRNA that was present in DC aggregates from untransplanted RAG^{-/-} mice or RAG^{-/-} mice shortly after T-cell transfer.

A CD103⁺ T-Cell Graft Gives Rise to CD103⁺ T Cells

CD103⁺ T_{reg} cells may develop in the host from naive donor T cells. Alternatively, donor CD103⁺ T_{reg} cells may expand in the adoptive host from the small (2 to 6%)²⁷

Figure 5. CD103⁺ CD25⁻ T cells colonize DC aggregates of transplanted mice. Double-immunofluorescence staining of large intestinal tissue of RAG^{-/-} mice obtained at day 14 after T-cell reconstitution was performed using anti-CD25 (green) together with anti-CD3 (red) or anti-CD103 (green) in combination with anti-CD3 (red). CD25⁺ cells are seen in ILAs (**box** in **A**). **B**: High magnification shows that CD25⁺ cells in the boxed area do not co-express CD3. Single-immunohistochemical staining reveals large numbers of CD25⁺ cells in DC aggregates of untransplanted RAG^{-/-} mice (**C**, **arrows**). Numerous CD103⁺ cells are present in DC aggregates (**box** in **D**) but are also found in the lamina propria outside these structures. At high magnification, a substantial number of CD103⁺ cells reveals co-expression of CD3 (**E**). Double-positive cells appear yellow/orange. **F**: The proportion of CD103⁺ T cells in DC aggregates and in the lamina propria was determined by counting ≥ 200 CD3⁺ cells per compartment in immunofluorescently stained tissue sections. Columns indicate the mean of three mice; error bars indicate the SD. The *P* value is given as determined by Student's *t*-test. **G**: From CD4⁺ lamina propria T cells (**top left**) of RAG^{-/-} mice at day 14 after reconstitution, CD103⁺ and CD103⁻ T cells were sorted (**top right**), and the purity of the CD103⁻ (**bottom left**) and CD103⁺ (**bottom right**) fractions was verified by reanalysis. **H**: The relative expression of Foxp3 was determined by quantitative RT-PCR and is indicated as the mean and SD of three measurements. The *P* value is given as determined by Student's *t*-test. A high Foxp3 expression is detectable only in the CD103⁺ fraction. Original magnifications: $\times 10$ (**A**, **C**, **D**); $\times 40$ (**B**, **E**).

Figure 6. TGF- β 1 expression in the large intestine and appearance of lamina propria CD103 $^{+}$ T $_{reg}$ cells in RAG $^{-/-}$ mice transplanted with CD103 $^{-}$ CD4 $^{+}$ T cells that suppress a Th1 response. **A:** The mRNA expression of TGF- β 1 was determined using HPRT as a housekeeping gene, as explained in the legend to Figure 3, and is given as the mean and SD of three measurements. *P* values are given as determined by Student's *t*-test. **Light columns** indicate the lamina propria, **dark columns** indicate DC aggregates. **B:** RAG $^{-/-}$ mice were reconstituted with unfractionated CD4 $^{+}$ T cells (CD103 $^{+/-}$ graft, **top**) and CD4 $^{+}$ T cells rigorously depleted of CD103 $^{+}$ T cells (CD103 $^{-}$ graft, **bottom**). After 2 weeks, lamina propria cells were isolated and the CD103 and Foxp3 expression was determined in the CD3 $^{+}$ CD4 $^{+}$ gate by flow cytometry analysis. High numbers of CD103 $^{+}$ T cells in the lamina propria of reconstituted mice were found independent of the CD103 $^{+}$ depletion status of the graft. A substantial fraction of CD103 $^{+}$ cells co-expresses Foxp3. **C:** Ovalbumin-specific transgenic CD4 $^{+}$ T cells were challenged with ovalbumin-pulsed spleen DCs in the presence of CD103 $^{+}$ or CD103 $^{-}$ T cells isolated from the lamina propria of T-cell reconstituted mice. Controls were performed with ovalbumin-pulsed or nonpulsed DCs in the absence of a suppressor T-cell population. IFN- γ production was determined in the supernatant by enzyme-linked immunosorbent assay and is shown as the mean and SD of three measurements. *P* values are given as determined by Student's *t*-test. CD103 $^{+}$, but not CD103 $^{-}$, T cells suppressed IFN- γ production. CD103 $^{+}$ T cells isolated from the lamina propria of mice reconstituted with unfractionated CD4 $^{+}$ T cells or with T cells depleted from CD103 $^{+}$ cells displayed similar inhibitory capacities.

CD103⁺ CD4⁺ T-cell subset present in the spleen-derived CD4⁺ T-cell graft from normal donor B6 mice. We reconstituted RAG^{-/-} mice with a CD4⁺ T-cell graft depleted of CD103⁺ cells to a purity of $\geq 99.5\%$ (data not shown). Colonic lamina propria T-cell populations in reconstituted mice contained similar numbers of CD103⁺ CD4⁺ T cells, irrespective of the CD103⁺ depletion status of the graft (Figure 6B). A substantial fraction of CD103⁺ T cells were Foxp3⁺ as determined by intracellular staining, whereas no Foxp3-expressing cells were detected in CD103⁻ T cells (Figure 6B). The proportion of Foxp3⁺ cells was not influenced by depleting the graft from CD103⁺ T cells. Hence, CD103⁺ CD4⁺ T_{reg} cells might be generated in the adoptive host.

CD103⁺ CD4⁺ T Cells Suppress a Th1 Response in Vitro

To confirm the regulatory nature of CD103⁺ CD3⁺ T cells, we assessed whether these cells were capable of inhibiting the conversion of naive CD4⁺ T cells into Th1 effector cells. To this end, we isolated spleen DCs from B6 mice and CD4⁺ T cells from OT-II mice that possess an ovalbumin-specific T-cell receptor. OT-II CD4⁺ T cells were challenged with either ovalbumin-pulsed or non-pulsed DCs (Figure 6C). Only ovalbumin-pulsed DCs in co-culture with OT-II CD4⁺ T cells showed a strong up-regulation of IFN- γ , indicating a Th1 polarization. IFN- γ induction was inhibited in the presence of CD103⁺ CD4⁺ T cells but not by CD103⁻ CD4⁺ T cells. CD103⁺ lamina propria T cells of RAG^{-/-} mice that had received an unfractionated or CD103 depleted T-cell graft, respectively, were equally potent in suppressing IFN- γ production.

CD103⁺ Foxp3⁺ T Cells Also Populate the Mesenteric Lymph Nodes and Spleen of Reconstituted Mice

To determine whether T_{reg} cells were restricted to the large intestine, or also colonized other lymphoid structures associated or unassociated with the intestinal mucosa, we isolated T cells from the mesenteric lymph nodes and the spleen of adoptively transferred mice with early IBD. Figure 7 demonstrates that these secondary lymphoid organs harbored a Foxp3⁺ fraction comprising ~10 to 20% of the CD4⁺ T cells. Foxp3⁺ cells almost exclusively resided within the CD103⁺ T-cell subset. Depletion of the T-cell graft from CD103⁺ cells had no significant impact on the abundance or phenotype of T_{reg} cells in the analyzed tissues. Thus, a similar proportion of CD103⁺ T_{reg} cells is present in the colonic lamina propria, mesenteric lymph node, and spleen of reconstituted RAG^{-/-} mice.

Discussion

A striking early event in the development of transfer colitis is the clustering of T cells in subepithelial DC aggregates of the colonic mucosa. Here, we demonstrate that DC aggregates contain a substantial proportion of c-kit⁺ and IL7-R⁺ cells. This, together with their number, distribution, and morphology, strongly suggest DC structures in RAG^{-/-} mice to be equivalents of ILAs of immunocompetent mice.¹⁰ It is difficult, however, to safely distinguish ILAs from organized lymphoid structures of the murine colon (colonic patches) in RAG^{-/-} mice. Colonic patches resemble Peyer's patches of the small intestine³¹ and characteristically contain several B-cell follicles, a feature that is not recognized in RAG^{-/-} mice and therefore not suitable to distinguish colonic patches from ILAs. Therefore, we cannot exclude the possibility that some of the larger DC aggregates actually represent colonic patches. The designation ILA comprises ILFs, crypto-

Figure 7. CD103⁺ Foxp3⁺ T cells are present in the mesenteric lymph nodes and spleen of T-cell reconstituted mice. T cells were isolated from the lamina propria of mice reconstituted with unfractionated CD4⁺ T cells or with T cells depleted from CD103⁺ cells. The expression of CD103 and Foxp3 was determined in the CD3⁺ CD4⁺ gate by flow cytometry analysis.

patches, and the large number of organized lymphoid structures displaying intermediate properties. So far, presumably the only undisputed function of ILAs is the maturation of B-cell responses in ILFs.^{12,32} The role of smaller ILA structures lacking fully developed B-cell follicles is obscure. It has been proposed that extrathymic development of T lymphocytes takes place in cryptopatches³³ but this hypothesis was questioned by recent findings.^{10,34,35} The data presented here suggest that ILAs may be involved in modulating T-cell responses of the intestinal immune system.

The high density of DCs in ILAs, their close association with the overlying epithelium, and their mature phenotype (as judged by the expression of MHC-II and co-stimulatory molecules) make them ideal candidates for priming T-cell responses to gut luminal antigens. Because Th1-biased reactions predominate in the transfer colitis model,³ we tested if the phenotype of DC aggregates can support initiation or promotion of Th1 T-cell responses.¹⁴ The expression of the proinflammatory cytokine IL-23, a potent signal in conferring Th1 polarization to naive T cells,³⁶ was marginal in ILAs and very low in the lamina propria of untransplanted mice and 2 weeks after T-cell transfer. DC aggregates may therefore have the potential to drive a Th1 reaction. However, IFN- γ , the main Th1 effector cytokine,³⁷ was found at only low levels in these organized lymphoid structures during the early phase of colitis, when high IFN- γ amounts were already present in the lamina propria. At advanced disease, substantial IFN- γ expression could also be observed in ILAs. Hence, ILAs do not appear to participate in mounting a Th1 reaction at a stage that is likely to be essential for the outcome of the immune response in this IBD model. This raises the question where the pathogenic Th1 reaction is initiated in this IBD model. Our data suggest that, although present at low densities, CD3⁺ T cells may receive Th1-inducing signals directly in the lamina propria. This seems difficult to be reconciled with the scarcity of CD11c⁺ DCs expressing co-stimulatory molecules in the lamina propria of untransplanted Rag^{-/-} mice. However, even a low number of DCs may be sufficient to cope with the few donor T cells that colonize the gut at the early phase of colitis. Another attractive candidate to induce

Th1 reactions in the lamina propria is the recently described population of CD70⁺ CD11c⁻ antigen-presenting cells.³⁸ These nonhematopoietic cells have been shown to drive a CD8⁺ T-cell response directed against *Listeria monocytogenes* in the intestinal mucosa. Malmström and colleagues³⁹ reported that Th1 effector cells could be generated in a CD134L-dependent mode by DCs residing in the mesenteric lymph nodes. CD134L⁺ DCs were detected even in untransplanted SCID mice, albeit in low numbers. Therefore, mesenteric lymph nodes might be the site of initial Th1 T-cell induction in the transfer colitis model in accordance with the well-recognized role of mesenteric lymph nodes in priming T-cell reactions to orally administered antigen.⁴⁰ With advancing disease, the abundance of IL-23 produced in the lamina propria, in conjunction with the large number of DCs present in the heavily inflamed gut mucosa,¹⁴ might become the major force to generate Th1 effector cells, thereby enhancing colitis in an autoamplifying manner. In addition to eliciting a Th1 reaction, an environment enriched in IL-23 could also support the generation of IL-17-expressing CD4⁺ effector T cells.⁴¹ Further studies are needed to address the question if and in what (micro)anatomical structure a Th17 reaction contributes to the development of IBD.

The transfer colitis model has been widely used to study the role of the lately identified T_{reg} cell population characterized by the expression of the transcription factor Foxp3.²⁴ This regulatory T-cell subset is able to prevent the development of IBD.²⁰ We observed increased numbers of Foxp3⁺ cells by immunohistochemistry and high Foxp3 mRNA expression in DC aggregates during IBD development, demonstrating that T_{reg} cells are present in these structures. Unexpectedly, we did not find appreciable numbers of T cells expressing CD25, the most widely used marker to detect T_{reg} cells.²⁴ In contrast, a significant fraction of T cells in DC aggregates expressed the $\alpha_E\beta_7$ integrin CD103. This molecule was recently shown to identify a subset of CD25⁻ FoxP3⁺ T_{reg} cells.²⁶ CD103 is expressed by a large fraction of T cells in the intestinal lamina propria of normal immunocompetent mice that are not regulatory T cells. To exclude that CD103 expression was merely indicative of a mucosa-

seeking phenotype rather than a regulatory function, we measured Foxp3 expression in CD103⁺ and CD103⁻ T cells isolated from the lamina propria of reconstituted mice. The observation that Foxp3 expression was substantially increased in the CD103⁺ T-cell fraction confirmed that T_{reg} cells are highly enriched within this subset. This was further underlined by the capacity of CD103⁺ T cells to suppress a Th1 response *in vitro*. The accumulation of CD25⁻ CD103⁺ T_{reg} cells in DC aggregates raises the question whether T_{reg} cells of this phenotype are selectively recruited into these structures. We consider this unlikely because CD103⁺ T_{reg} cells have been shown to preferentially migrate into inflamed sites.²⁶ However, T_{reg} cells started to appear in DC aggregates at an early phase of IBD before overt inflammation was histologically manifest. Moreover, as we show here, depleting CD103⁺ cells from the T-cell graft did not reduce the number of CD103⁺ Foxp3⁺ T cells in the lamina propria of reconstituted mice. This indicates that these T_{reg} cells might either expand in the adoptive host or differentiate from noncommitted T cells. It is tempting to speculate that a conversion of naive CD4⁺ T cells into CD25⁻ CD103⁺ T_{reg} cells is facilitated by the environment of DC aggregates. Although some T_{reg} cell subsets are of thymic origin⁴² there is increasing evidence that Foxp3⁺ regulatory T cells are also induced extrathymically.^{29,43–45} In one report, conventional CD4⁺ T cells could be converted *in vitro* into T_{reg} cells under the influence of TGF- β .²⁹ TGF- β has also been shown to induce CD103 expression.^{30,46} According to a recently proposed model, CD103⁺ T_{reg} cells may be generated in peripheral tissues on encounter with cognate antigen.²⁶ The high level of TGF- β expression in ILAs of untransplanted RAG^{-/-} mice, together with the prevalence of DCs with a mature phenotype that have access to luminal antigen may facilitate the local generation of CD25⁻ CD103⁺ T_{reg} cells in these organized lymphoid structures. Subsequently, CD103⁺ T_{reg} cells could be exported from ILAs to the lamina propria.

Although the generation of T_{reg} cells from nonregulatory precursors in a supportive environment of the host is an intriguing possibility, we cannot exclude an expansion of pre-existing regulatory T cells differentiated in the donor thymus. Despite rigorously clearing the graft from CD103⁺ T cells, low numbers of T_{reg} cells are likely to be carried over into the recipient animal. After proliferation, these may account for the relatively high number of T_{reg} cells observed in the early phase of IBD development. That expansion rather than differentiation may be the predominant mechanism to provide T_{reg} cells in the transfer colitis model is suggested by a recent study using donor mice with a red fluorescent protein knocked into the endogenous Foxp3 locus.⁴⁷

CD103⁺ Foxp3⁺ T_{reg} cells were not restricted to the large intestine but were also present in the mesenteric lymph nodes and the spleen at similar densities as in the gut. This indicates that different types of secondary lymphatic tissues are able to support the differentiation and/or expansion of T_{reg} cells and thus perform a similar function as large intestinal DC clusters. Alternatively, T_{reg} cells generated in DC aggregates of the large gut may

recirculate and colonize extra-intestinal secondary lymphoid organs. The migratory behavior of T_{reg} cells and the involvement of extraintestinal secondary lymphoid organs in regulatory T-cell reactions are still incompletely understood and will have to be addressed in future studies.

It is unresolved why severe IBD develops despite the presence of CD103⁺ T_{reg} cells that can suppress this experimental colitis²⁷ and inhibit a Th1 response *in vitro*. Our data suggest that the number of T_{reg} cells generated is insufficient to down modulate the colitogenic T-cell responses because we found only a few Foxp3⁺ cells and low levels of Foxp3 mRNA in the severely inflamed lamina propria. That large T_{reg} numbers might be critical is illustrated by the relatively high ratios of CD103⁺ T cells to naive T cells (1:3) that were required to prevent colitis in co-transfer experiments.²⁷

Taken together, the data presented here suggest that large intestinal DC aggregates participate in the generation of T_{reg} cells in a murine model of IBD. Organized lymphoid structures of the colon are not confined to this murine system because they have been described as a histomorphological hallmark of human ulcerative colitis.⁴⁸ Further studies are needed to determine whether regulatory T cells are present in ILAs of human IBD.

Acknowledgment

We thank Ellen Allmendinger for expert technical assistance.

References

1. Bouma G, Strober W: The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 2003, 3:521–533
2. Strober W, Fuss IJ, Blumberg RS: The immunology of mucosal models of inflammation. *Annu Rev Immunol* 2002, 20:495–549
3. Powrie F, Leach MW, Mauze S, Menon S, Caddle LB, Coffman RL: Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RB^{hi} CD4⁺ T cells. *Immunity* 1994, 1:553–562
4. Morrissey PJ, Charrier K, Braddy S, Liggitt D, Watson JD: CD4⁺ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified CD4⁺ T cells. *J Exp Med* 1993, 178:237–244
5. Powrie F, Correa-Oliveira R, Mauze S, Coffman RL: Regulatory interactions between CD45RB^{high} and CD45RB^{low} CD4⁺ T cells are important for the balance between protective and pathogenic cell-mediated immunity. *J Exp Med* 1994, 179:589–600
6. Claesson MH, Bregenholt S, Bonhagen K, Thoma S, Möller P, Grusby MJ, Leithäuser F, Nissen MH, Reimann J: Colitis-inducing potency of CD4⁺ T cells in immunodeficient, adoptive hosts depends on their state of activation, IL-12 responsiveness, and CD45RB surface phenotype. *J Immunol* 1999, 162:3702–3710
7. Powrie F, Mauze S, Coffman RL: CD4⁺ T-cells in the regulation of inflammatory responses in the intestine. *Res Immunol* 1997, 148:576–581
8. Mowat AM: Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* 2003, 3:331–341
9. Brandtzaeg P, Pabst R: Let's go mucosal: communication on slippery ground. *Trends Immunol* 2004, 25:570–577
10. Pabst O, Herbrand H, Worbs T, Friedrichsen M, Yan S, Hoffmann MW, Korner H, Bernhardt G, Pabst R, Förster R: Cryptopatches and isolated lymphoid follicles: dynamic lymphoid tissues dispensable for

- the generation of intraepithelial lymphocytes. *Eur J Immunol* 2004, 35:98–107
11. Kanamori Y, Ishimaru K, Nanno M, Maki K, Ikuta K, Nariuchi H, Ishikawa H: Identification of novel lymphoid tissues in murine intestinal mucosa where clusters of c-kit⁺ IL-7R⁺ Thy1⁺ lympho-hemopoietic progenitors develop. *J Exp Med* 1996, 184:1449–1459
  12. Hamada H, Hiroi T, Nishiyama Y, Takahashi H, Masunaga Y, Hachimura S, Kaminogawa S, Takahashi-Iwanaga H, Iwanaga T, Kiyono H, Yamamoto H, Ishikawa H: Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J Immunol* 2002, 168:57–64
  13. Lorenz RG, Chaplin DD, McDonald KG, McDonough JS, Newberry RD: Isolated lymphoid follicle formation is inducible and dependent upon lymphotoxin-sufficient B lymphocytes, lymphotoxin β receptor, and TNF receptor I function. *J Immunol* 2003, 170:5475–5482
  14. Leithäuser F, Trobonjaca Z, Möller P, Reimann J: Clustering of colonic lamina propria CD4⁺ T cells to subepithelial dendritic cell aggregates precedes the development of colitis in a murine adoptive transfer model. *Lab Invest* 2001, 81:1339–1349
  15. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE: RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 1992, 68:869–877
  16. Barnden MJ, Allison J, Heath WR, Carbone FR: Defective TCR expression in transgenic mice constructed using cDNA-based α - and β -chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol* 1998, 76:34–40
  17. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y: 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* 1997, 407:313–319
  18. Krajina T, Leithäuser F, Reimann J: MHC class II-independent CD25⁺ CD4⁺ CD8 $\alpha\beta$ ⁺ $\alpha\beta$ T cells attenuate CD4⁺ T cell-induced transfer colitis. *Eur J Immunol* 2004, 34:705–714
  19. Leithäuser F, Trobonjaca Z, Reimann J, Möller P: In situ characterization of genetically targeted (green fluorescent) single cells and their microenvironment in an adoptive host. *Am J Pathol* 2001, 158:1975–1983
  20. Hori S, Nomura T, Sakaguchi S: Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003, 299:1057–1061
  21. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001, 29:e45
  22. Iwasaki A, Kelsall BL: Unique functions of CD11b⁺, CD8 α ⁺, and double-negative Peyer's patch dendritic cells. *J Immunol* 2001, 166:4884–4890
  23. Steinman RM, Nussenzweig MC: Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci USA* 2002, 99:351–358
  24. Fehervari Z, Sakaguchi S: CD4⁺ Tregs and immune control. *J Clin Invest* 2004, 114:1209–1217
  25. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY: Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 2005, 22:329–341
  26. Huehn J, Siegmund K, Lehmann JC, Siewert C, Haubold U, Feuerer M, Debes GF, Lauber J, Frey O, Przybylski GK, Niesner U, de la Rosa M, Schmidt CA, Brauer R, Buer J, Scheffold A, Hamann A: Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4⁺ regulatory T cells. *J Exp Med* 2004, 199:303–313
  27. Lehmann J, Huehn J, de la Rosa M, Maszyra F, Kretschmer U, Krenn V, Brunner M, Scheffold A, Hamann A: Expression of the integrin α E β 7 identifies unique subsets of CD25⁺ as well as CD25[−] regulatory T cells. *Proc Natl Acad Sci USA* 2002, 99:13031–13036
  28. McHugh RS, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M, Byrne MC: CD4⁺CD25⁺ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 2002, 16:311–323
  29. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM: Conversion of peripheral CD4⁺ CD25[−] naive T cells to CD4⁺ CD25⁺ regulatory T cells by TGF- β induction of transcription factor Foxp3. *J Exp Med* 2003, 198:1875–1886
  30. Kilshaw PJ, Murrant SJ: A new surface antigen on intraepithelial lymphocytes in the intestine. *Eur J Immunol* 1990, 20:2201–2207
  31. Dohi T, Fujihashi K, Rennert PD, Iwatani K, Kiyono H, McGhee JR: Hapten-induced colitis is associated with colonic patch hypertrophy and T helper cell 2-type responses. *J Exp Med* 1999, 189:1169–1180
  32. Shikina T, Hiroi T, Iwatani K, Jang MH, Fukuyama S, Tamura M, Kubo T, Ishikawa H, Kiyono H: IgA class switch occurs in the organized nasopharynx- and gut-associated lymphoid tissue, but not in the diffuse lamina propria of airways and gut. *J Immunol* 2004, 172:6259–6264
  33. Saito H, Kanamori Y, Takemori T, Nariuchi H, Kubota E, Takahashi-Iwanaga H, Iwanaga T, Ishikawa H: Generation of intestinal T cells from progenitors residing in gut cryptopatches. *Science* 1998, 280:275–278
  34. Eberl G, Marmon S, Sunshine MJ, Rennert PD, Choi Y, Littman DR: An essential function for the nuclear receptor ROR γ t in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol* 2004, 5:64–73
  35. Guy-Grand D, Azogui O, Celli S, Darce S, Nussenzweig MC, Kourilsky P, Vassalli P: Extrathymic T cell lymphopoiesis: ontogeny and contribution to gut intraepithelial lymphocytes in athymic and euthymic mice. *J Exp Med* 2003, 197:333–341
  36. Trinchieri G, Pflanz S, Kastelein RA: The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity* 2003, 19:641–644
  37. Robinson DS, O'Garra A: Further checkpoints in Th1 development. *Immunity* 2002, 16:755–758
  38. Laouar A, Haridas V, Vargas D, Zhinan X, Chaplin D, van Lier RA, Manjunath N: CD70⁺ antigen-presenting cells control the proliferation and differentiation of T cells in the intestinal mucosa. *Nat Immunol* 2005, 6:698–706
  39. Malmström V, Shipton D, Singh B, Al Shamkhani A, Puklavec MJ, Barclay AN, Powrie F: CD134L expression on dendritic cells in the mesenteric lymph nodes drives colitis in T cell-restored SCID mice. *J Immunol* 2001, 166:6972–6981
  40. Smith KM, Davidson JM, Garside P: T-cell activation occurs simultaneously in local and peripheral lymphoid tissue following oral administration of a range of doses of immunogenic or tolerogenic antigen although tolerized T cells display a defect in cell division. *Immunology* 2002, 106:144–158
  41. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT: Interleukin 17-producing CD4⁺ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005, 6:1123–1132
  42. Sakaguchi S: The origin of FOXP3-expressing CD4⁺ regulatory T cells: thymus or periphery. *J Clin Invest* 2003, 112:1310–1312
  43. Apostolou I, von Boehmer H: In vivo instruction of suppressor commitment in naive T cells. *J Exp Med* 2004, 199:1401–1408
  44. Curotto de Lafaille MA, Lino AC, Kutchukhidze N, Lafaille JJ: CD25[−] T cells generate CD25⁺Foxp3⁺ regulatory T cells by peripheral expansion. *J Immunol* 2004, 173:7259–7268
  45. Liang S, Alard P, Zhao Y, Parnell S, Clark SL, Kosiewicz MM: Conversion of CD4⁺ CD25[−] cells into CD4⁺ CD25⁺ regulatory T cells in vivo requires B7 costimulation, but not the thymus. *J Exp Med* 2005, 201:127–137
  46. Parker CM, Cepek KL, Russell GJ, Shaw SK, Posnett DN, Schwarting R, Brenner MB: A family of β 7 integrins on human mucosal lymphocytes. *Proc Natl Acad Sci USA* 1992, 89:1924–1928
  47. Wan YY, Flavell RA: Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc Natl Acad Sci USA* 2005, 102:5126–5131
  48. Yeung MM, Melgar S, Baranov V, Oberg A, Danielsson A, Hammarström S, Hammarström ML: Characterisation of mucosal lymphoid aggregates in ulcerative colitis: immune cell phenotype and TCR $\gamma\delta$ expression. *Gut* 2000, 47:215–227