Gut memories do not fade: epigenetic regulation of lasting gut homing receptor expression in CD4$^+$ memory T cells

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The concept of a "topographical memory" in lymphocytes implies a stable expression of homing receptors mediating trafficking of lymphocytes back to the tissue of initial activation. However, a significant plasticity of the gut-homing receptor $\alpha_4\beta_7$ was found in CD8$^+$ T cells, questioning the concept. We now demonstrate that $\alpha_4\beta_7$ expression in murine CD4$^+$ memory T cells is, in contrast, imprinted and remains stable in the absence of the inducing factor retinoic acid (RA) or other stimuli from mucosal environments. Repetitive rounds of RA treatment enhanced the stability of *de novo* induced $\alpha_4\beta_7$. A novel enhancer element in the murine *Itga4* locus was identified that showed, correlating to stability, selective DNA demethylation in mucosa-seeking memory cells and methylation-dependent transcriptional activity in a reporter gene assay. This implies that epigenetic mechanisms contribute to the stabilization of $\alpha_4\beta_7$ expression. Analogous DNA methylation patterns could be observed in the human *ITGA4* locus, suggesting that its epigenetic regulation is conserved between mice and men. These data prove that mucosa-specific homing mediated by $\alpha_4\beta_7$ is imprinted in CD4$^+$ memory T cells, reinstating the validity of the concept of "topographical memory" for mucosal tissues, and imply a critical role of epigenetic mechanisms.

INTRODUCTION

Long-term immunity relies on memory cells providing protection against recurring infections. The shaping of functional features of memory cells during primary reactions includes the imprinting of migration properties that secure their optimal targeting to potentially affected compartments. This topographical memory, described as organ-specific *homing* more than 50 years ago,[1] was a well-accepted paradigm that appeared to be supported by a number of studies on gut- and skin-specific homing of T memory cells (T_{mem}) generated in site-specific immune responses.[2–4]

Homing of T cells into distinct tissues depends on the expression of adhesion molecules and chemokine receptors. Naive CD4$^+$ T cells (T_N) uniformly recirculate through secondary lymphoid organs via L-selectin and CCR7, a behavior preserved by early stages of T_{mem}. However, upon further differentiation, T cells home preferentially into peripheral tissues such as the skin and gut or into inflamed sites. Eventually, part of the cells will become tissue-resident cells that stop circulating.[5,6] In this study, we investigated the stability and molecular regulation of integrin $\alpha_4\beta_7$-dependent mucosa-specific homing of CD4$^+$ T_{mem} from lymphoid tissues that largely belong to the recirculating pool of CD4$^+$ T_{mem}.

T cells homing to skin express E- and P-selectin ligands and the chemokine receptor CCR4 and/or CCR8. By contrast, gut-homing T_{mem} selectively express the integrin $\alpha_4\beta_7$ and partially express the chemokine receptor CCR9, enabling their entry into the intestine and associated lymphoid tissues by interacting with the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and the chemokine CCL25, respectively.[7–9] Expression of these receptors is *de novo* induced or upregulated upon antigen-induced activation within mesenteric lymph nodes (mLNs) or

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Peyer's patches.[10] The vitamin A metabolite retinoic acid (RA), provided by CD103$^+$ dendritic cells (DCs) and/or stromal cells in mLNs,[11,12] has been identified as a crucial factor together with T-cell activation for induction of $\alpha_4\beta_7$ and CCR9.[13]

Notably, the assumption of the homing paradigm that T_{mem} cells acquire a stable homing phenotype by imprinted expression of a given set of adhesion receptors in CD4$^+$ T_{mem} only has been proven experimentally for selectin ligands so far.[14,15] In fact, recent studies in CD8$^+$ T cells have rather demonstrated that the mucosal homing receptor $\alpha_4\beta_7$ is only transiently expressed on CD8$^+$ T cells and that $\alpha_4\beta_7^+$ gut-homing CD8$^+$ T cells might convert into a skin-homing phenotype.[16–18] This implied plasticity of migratory phenotypes and the possibility that certain homing phenotypes require continuous stimulation by environmental signals to be maintained.

In the first part of this study, we therefore investigated whether CD4$^+$ T_{mem} retain expression of the gut-homing receptor $\alpha_4\beta_7$ *in vivo* or upon restimulation *in vitro*, or whether continuous instruction by tissue-localized factors is required for the maintenance of gut-homing properties. Indeed, *ex vivo* isolated $\alpha_4\beta_7^+$ CD4$^+$ T_{mem} maintained their initial $\alpha_4\beta_7$ expression level independent of the presence of RA or other environmental factors encountered during recirculation through gut or gut-associated lymphoid tissues (GALTs). To acquire a stable phenotype, repeated stimulation of T_N cells in the presence of RA was required, suggesting a stepwise acquisition of an imprinted state.

In a second part, we analyzed the molecular basis for the imprinted phenotype. The regulation of α_4-integrins is not well understood, despite significant medical relevance of α_4-integrins as a target for antibody therapies in chronic inflammatory bowel disease and multiple sclerosis.

Here we focused on the role of epigenetic mechanisms, as a growing list of evidence suggests that DNA methylation or histone modifications might be the key for a stably imprinted expression pattern in polarized T-helper (Th) subsets, stable lineages such as regulatory T cells, expression of signature cytokines, or master transcription factors.[19–22] Albeit it appears suggestive, homing receptors have hardly been analyzed in this respect so far.

While some histone modifications appear to be solely associated with current transcriptional activity, notably DNA methylation and other repressive mechanisms are key candidates for the regulation of a long-term, heritable phenotype.[23] We therefore analyzed the DNA methylation patterns of the *Itga4* locus—coding for the integrin α_4 chain—in murine and human T cell subsets. Indeed, differential DNA methylation was observed in distinct regulatory regions and appears to control the stability of *Itga4* expression.

RESULTS

Stable expression of $\alpha_4\beta_7$ on murine CD4$^+$ memory T cells *in vivo*

First, the stability of $\alpha_4\beta_7$ expression on murine CD4$^+$ T_{mem} was analyzed *in vivo*. For this, we labeled *ex vivo* isolated, highly pure $\alpha_4\beta_7^+$ and $\alpha_4\beta_7^-$ CD45RB^{low} CD4$^+$ T_{mem} subsets (**Supplementary Figure 1A** online) with CFSE, transferred them into recipient mice and reanalyzed $\alpha_4\beta_7$ expression on transferred cells 15 days (**Figure 1a** and **b**) or 6 weeks later (**Figure 1c**). The majority of $\alpha_4\beta_7^+$ CD4$^+$ T_{mem} maintained high $\alpha_4\beta_7$ expression for at least 6 weeks, whereas transferred $\alpha_4\beta_7^-$ CD4$^+$ T_{mem} remained negative for $\alpha_4\beta_7$. These data show that the expression of $\alpha_4\beta_7$ in CD4$^+$ T_{mem} is stable *in vivo*. Interestingly, some $\alpha_4\beta_7^+$ CD4$^+$ T_{mem} were also recovered

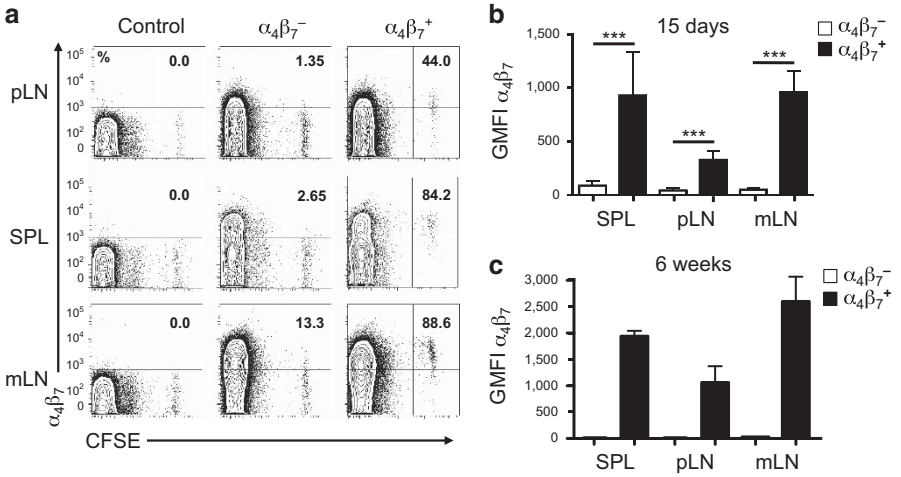

Figure 1 Stable expression of integrin $\alpha_4\beta_7$ on *ex vivo* isolated CD4$^+$ T_{mem}. (**a** and **b**) $\alpha_4\beta_7$ Expression on T_{mem} 15 days after adoptive transfer. *Ex vivo* isolated $\alpha_4\beta_7^+$ P-lig$^-$CD4$^+$CD45RB^{low} and $\alpha_4\beta_7^-$ P-lig$^-$CD4$^+$CD45RB^{low} T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and transferred into recipient mice. Fifteen days after transfer, cells from peripheral lymph node (pLN), mesenteric lymph node (mLN), and spleen (SPL) were reanalyzed for $\alpha_4\beta_7$ expression. Representative FACS (fluorescence-activated cell sorter) plots from three independent experiments are shown in (**a**). Control: staining control FMO-$\alpha_4\beta_7$ (fluorescence minus one). Numbers indicate frequencies of $\alpha_4\beta_7^+$ cells in transferred CFSE$^+$ cells. Accumulated values from three independent experiments ($\alpha_4\beta_7^-$, $n=6$; $\alpha_4\beta_7^+$, $n=9$) are shown in (**b**) as mean ± s.d. of pooled geometric mean fluorescence intensity (GMFI) of CFSE+ cells. (**c**) $\alpha_4\beta_7$ Expression 6 weeks after transfer. Mean ± s.d. of pooled GMFI data from three or four mice in three experiments. Student's *t*-test with ***$P<0.001$.

from the subcutaneous peripheral lymph nodes (pLNs), albeit with reduced levels of retained $\alpha_4\beta_7$ compared with the spleen or mLN. This might be due to the heterogeneity of the transferred CD45RB^{low} CD4$^+$ population, which contains, besides $\alpha_4\beta_7^+$ effector/memory cells, central memory T cells (**Supplementary Figure 1A** and **B**), which coexpress other homing receptors such as L-selectin (CD62L). L-selectin controls pLN immigration and is likely to cause enrichment of the CD62L$^+$ fraction at this site.

Stable expression of $\alpha_4\beta_7$ on murine CD4$^+$ T_{mem} depends neither on stabilizing factors within the GALT nor on the presence of RA

The observed *in vivo* stability could be mediated either by a cell-intrinsic imprinting of $\alpha_4\beta_7$ expression or by continual encounter with environmental signals required for its maintenance. As $\alpha_4\beta_7$ is induced by the vitamin A metabolite RA,[13] we asked whether the maintained expression of $\alpha_4\beta_7$ *in vivo* depends on the presence of RA.

To investigate this question, we generated vitamin A-deficient mice by pre- and postnatal feeding of a vitamin A-deficient diet for at least 14 weeks and used them as recipients for adoptive transfer of $\alpha_4\beta_7^+$ and $\alpha_4\beta_7^-$ CD4$^+$ T_{mem}. As shown in **Figure 2a**, the expression of $\alpha_4\beta_7$ on transferred CD4$^+$ T_{mem} was not affected by vitamin A deficiency. By contrast, endogenous T cells displayed low levels of $\alpha_4\beta_7$ under these conditions (**Figure 2a**), and naive OVA-specific CD4$^+$ T cells transferred into vitamin A-deficient mice failed to upregulate $\alpha_4\beta_7$ and CCR9 upon oral application of OVA protein and cholera toxin (**Figure 2b**). This proved the effective removal of RA from the system. The data indicate that while RA is critical for the *de novo* induction of $\alpha_4\beta_7$, it is not required for the maintenance of $\alpha_4\beta_7$ on CD4$^+$ T_{mem}.

T_{mem} residing in secondary lymphoid tissues are known to recirculate to some extent between blood, lymph, and tissues.[24,25] We therefore asked whether local factors in the gut or GALT were required for the stability of $\alpha_4\beta_7$ expression in CD4$^+$ T_{mem}. To prevent access of CD4$^+$ T_{mem} to mucosal sites, we blocked the adhesion molecules MAdCAM-1 and vascular cell adhesion molecule-1 (VCAM-1) involved in the entry of lymphocytes into this compartment by injection of anti-MAdCAM-1 and anti-VCAM-1 antibodies. This treatment reduced the entry of CD4$^+$ T_{mem} into both gut and GALT by 85–95% (**Supplementary Figure 2**). To examine whether the maintenance of $\alpha_4\beta_7$ expression depends on gut-derived instructive signals, we transferred $\alpha_4\beta_7^+$ CD4$^+$ T_{mem} into recipient mice together with injection of either anti-MAdCAM-1 plus anti-VCAM-1 antibodies or a rat-immunoglobulin G (IgG) control antibody. Antibody treatment was repeated every 3 days until analysis on day 15, resulting in the exclusion of transferred CD4$^+$ T_{mem} from the gut and GALT and their accumulation in the spleen (**Figure 2c** and **Supplementary Figure 2**). These conditions did not result in any reduction of $\alpha_4\beta_7$ expression on transferred $\alpha_4\beta_7^+$ CD4$^+$ T_{mem} (**Figure 2c**), suggesting that the maintenance of $\alpha_4\beta_7$ expression, in contrast to its induction on T_N cells, is independent of signals provided within the gut and GALT, but relies on a cell-intrinsic mechanism.

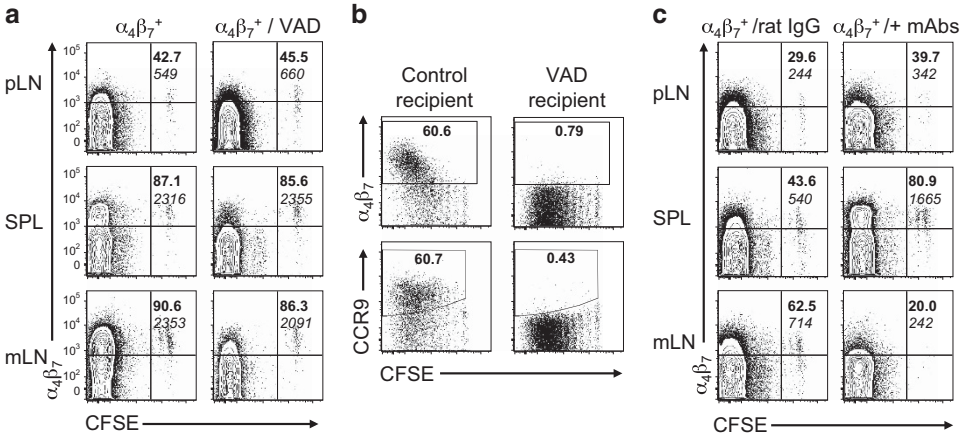

Figure 2 Stable expression of integrin $\alpha_4\beta_7$ on *ex vivo* isolated T_{mem} after transfer into vitamin A-deficient mice or exclusion from gut-associated lymphoid tissues (GALT). (**a**) Expression is retained in the absence of vitamin A. *Ex vivo* isolated $\alpha_4\beta_7^+$ P-lig$^-$CD4$^+$CD45RB^{low} and $\alpha_4\beta_7^-$ P-lig$^-$CD4$^+$CD45RB^{low} T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and transferred into control or vitamin A-depleted (VAD) mice. After 15 days, transferred cells were reanalyzed for $\alpha_4\beta_7$ expression. At 15 days after transfer, cells from peripheral lymph node (pLN), mesenteric lymph node (mLN) and spleen (SPL) were isolated and reanalyzed for $\alpha_4\beta_7$ expression. Representative FACS (fluorescence-activated cell sorter) plots from three independent experiments with eight mice; the frequency of $\alpha_4\beta_7^+$ cells (bold) and geometric mean fluorescence index (GMFI) (italic) among transferred cells is given. (**b**) Lack of $\alpha_4\beta_7$ and CCR9 upregulation verifies depletion of vitamin A. CFSE-labeled Ova-specific CD4$^+$CD62L^{high} T cells from DO11.10 mice were injected into control or VAD mice. At 24 h after transfer, mice were immunized with Ova protein and cholera toxin by oral gavage. On day 4 after immunization, analysis of $\alpha_4\beta_7$ expression on OVA-specific (KJ71-16$^+$) cells was performed by flow cytometry. Plots are gated on transferred KJ$^+$ cells. (**c**) Expression of $\alpha_4\beta_7$ is retained upon exclusion from GALT. *Ex vivo* FACS-sorted $\alpha_4\beta_7^+$ P-lig$^-$CD4$^+$CD45RB^{low} and $\alpha_4\beta_7^-$ P-lig$^-$CD4$^+$CD45RB^{low} T cells were labeled with CFSE and transferred into recipient mice. To exclude $\alpha_4\beta_7^+$ T_{mem} from GALT recipient mice received anti-MAdCAM-1 (Meca367; 500 μg per mouse) and anti-VCAM-1 Abs (6C7.1; 200 μg per mouse) or, as control, rat IgG intravenously at the day of cell transfer and then every 3 days until the day of analysis. Representative FACS plot from three independent experiments with four mice; the frequency of $\alpha_4\beta_7^+$ cells and GMFI among transfered CFSE$^+$ cells is given.

$\alpha_4\beta_7$, but not CCR9, displays stability on murine T_{mem} during *in vitro* culture

We tested *ex vivo* sorted $\alpha_4\beta_7^+$ $CD4^+$ T_{mem} for their phenotypic stability in *in vitro* cultures and observed that they largely retained $\alpha_4\beta_7$ expression upon restimulation (**Figure 3a**), confirming the imprinted state of $\alpha_4\beta_7$ in memory cells. Notably, expression was retained not only in the absence of RA but also in the presence of interleukin-12 (IL-12), which is known to upregulate the competing homing receptors P- and E-selectin ligands ("skin-homing phenotype").[26,27] Selectin ligands are expressed in T_{mem}, but, for *ex vivo* isolated cells, in a largely mutually exclusive way to $\alpha_4\beta_7$ (see **Figure 6** and **Supplementary Figure 1**).

Besides $\alpha_4\beta_7$, the chemokine receptor CCR9 is also induced by RA. CCR9 is thought to have a role in the immigration of cells to the small intestine; however, in accordance with previous reports,[16] only a small fraction of—mostly $\alpha_4\beta_7^+$—$CD4^+$ T_{mem} from mLNs and spleen express this receptor. When *ex vivo*-sorted $CCR9^+$ T_{mem} were stimulated *in vitro*, CCR9 was almost completely lost in the absence but retained in the presence of RA (**Figure 3b**), suggesting that CCR9 is not imprinted in $CD4^+$ T_{mem} but requires continual RA signals from the environment to remain expressed. These data add further evidence to previous findings, suggesting that the regulation of the gut-homing receptors $\alpha_4\beta_7$ and CCR9 differs.[28,29]

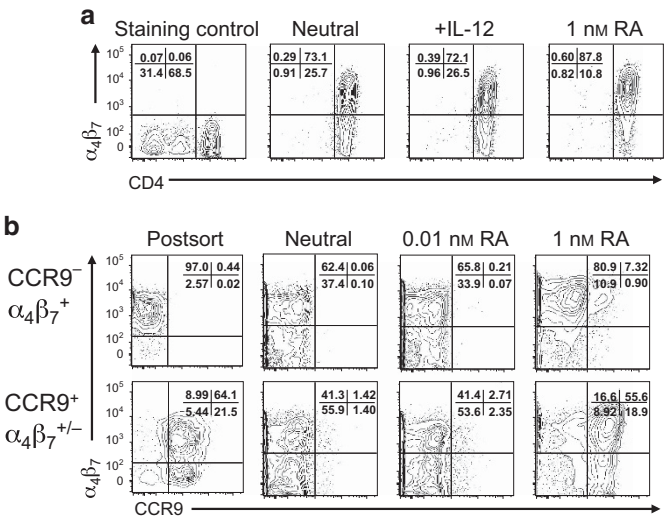

Figure 3 Integrin $\alpha_4\beta_7$, but not CCR9, expression is stably imprinted within *ex vivo* isolated T_{mem}. (**a**) *Ex vivo* isolated $\alpha_4\beta_7^+$ P-lig$^-$ $CD4^+$ $CD45RB^{low}$ T cells were stimulated with plate-bound anti-CD3/28 antibodies and interleukin-2 (IL-2) in the presence or absence of retinoic acid (RA) (1 nM) and IL-12 (5 ng ml^{-1}). On day 5, cells were harvested and analyzed for homing receptor expression by flow cytometry. Some unstained cells (lower left quadrant) were added in the staining control to facilitate compensation. (**b**) *Ex vivo* isolated $CCR9^-$ $\alpha_4\beta_7^+$ and $CCR9^+$ $\alpha_4\beta_7^{+/-}$ T cells (P-lig$^-$ $CD4^+$ $CD45RB^{low}$) were stimulated with plate-bound anti-CD3/28 antibodies and IL-2 in the presence or absence of RA (0.1 and 1 nM). On day 5, cells were analyzed for $\alpha_4\beta_7$ and CCR9 expression. FACS (fluorescence-activated cell sorter) plots are representative of three independent experiments each performed in (**a**) triplicates or (**b**) duplicates.

Repetitive, but not single, stimulation in the presence of RA stabilizes $\alpha_4\beta_7$ expression

$\alpha_4\beta_7$ Expression can be induced in $CD4^+$ T cells by RA, but it is unclear whether this is sufficient to achieve stable $\alpha_4\beta_7$ expression as found in $CD4^+$ T_{mem}. Therefore, we induced $\alpha_4\beta_7$ expression on murine $CD25^-CD4^+CD62L^+$ T_N cells during *in vitro* activation in the presence of RA and anti-IL-4 antibody (IL-4 has been reported to influence negatively $\alpha_4\beta_7$ induction *in vitro*).[16] On days 5 and 11 of culture, the cells were restimulated in the absence or presence of RA. $\alpha_4\beta_7$ Expression was analyzed at days 5, 11, and 17. As expected, T-cell activation in the presence of RA resulted in the efficient upregulation of $\alpha_4\beta_7$ (**Figure 4a** and **b**). However, *in vitro*-generated $\alpha_4\beta_7^+$ T cells rapidly lost $\alpha_4\beta_7$ expression when restimulated for 5 days in the absence of RA. Only $\alpha_4\beta_7^+$ T cells that had been stimulated two times in the presence of RA maintained $\alpha_4\beta_7$ expression upon restimulation in the absence of RA (**Figure 4a** and **b**). Thus, repetitive stimulation with RA, in contrast to single treatment, was sufficient to stabilize $\alpha_4\beta_7$ expression *in vitro*, suggesting that imprinting of $\alpha_4\beta_7$ expression in T cells requires a repeated or prolonged encounter with instructive signals.

Consistent with the *in vitro* stability, repeatedly RA-treated T cells retained a higher expression of $\alpha_4\beta_7$ compared with control cells on day 15 after transfer into recipient mice (**Figure 4c**), albeit the expression level was lower than at the time of transfer. This indicates that repetitive rounds of stimulation in the presence of RA resulted in at least a partial stabilization of $\alpha_4\beta_7$ expression in T cells.

Two regulatory elements in the murine *Itga4* locus show epigenetic modifications correlating with the stability of $\alpha_4\beta_7$ expression

Cell-intrinsic imprinting of a given gene activity state often relies on mechanisms of epigenetic regulation such as DNA methylation of critical genomic elements. We therefore performed a pilot MeDIP-on-Chip experiment to search for potential differentially methylated regions in the *Itga4* and *Itgb7* loci in *ex vivo* $\alpha_4\beta_7^+$ vs. $\alpha_4\beta_7^-$ $CD4^+$ T_{mem} subsets (data not shown). Differential methylation was apparent in two regions in the *Itga4* locus: the *Itga4* promoter upstream of the transcription start site containing a CpG-island (CpG-I), and a region in the second intron, which we termed differentially methylated region 1 (dmr1), both containing several CpG motifs (**Figure 5a**). No dmr were observed in the *Itgb7* locus. In line with previous reports,[30] we found only *Itga4*, but not *Itgb7*, to be regulated by repetitive RA treatment (**Supplementary Figure 3**). For these reasons, we focused our efforts on clarifiying the mechanism for the stability of $\alpha_4\beta_7$ expression on the *Itga4* locus.

We tested both regions in the *Itga4* gene for their regulatory activity on transcription using a classic luciferase reporter assay in murine $CD4^+$ T cells (**Figure 5b** and **c**). As expected, the *Itga4* promoter (but not dmr1; **Figure 5b**) was able to induce expression of the luciferase reporter, whereas the dmr1 element displayed an enhancer function when tested in conjunction with a promoter (**Figure 5c**).

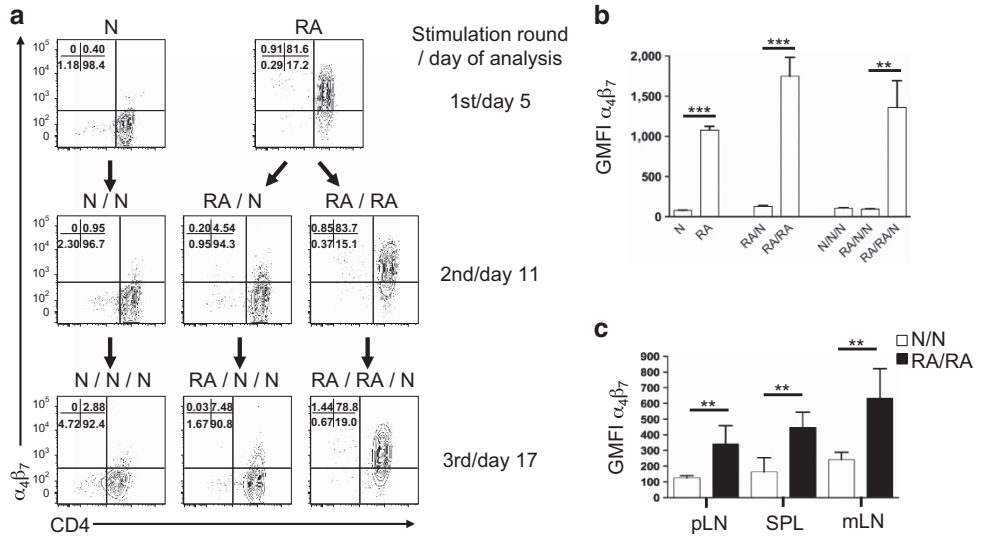

Figure 4 Repetitive, but not single, stimulation in the presence of retinoic acid (RA) stabilizes $\alpha_4\beta_7$ expression on CD4$^+$ T_N cells *in vitro*. (**a** and **b**) CD4$^+$ T_N cells were TCR stimulated (1st round) *in vitro* with plate-bound anti-CD3/CD28 antibodies under neutral ("N" = no further supplements) or polarizing conditions with RA (1 nM) and anti-IL-4 antibody ("RA"). TCR stimulation with one of the two conditions was repeated on day 5 (2nd round) and on day 11 (3rd round). Cells were analyzed for $\alpha_4\beta_7$ expression before restimulation on day 5, 11, and 17. (**a**) Representative FACS (fluorescence-activated cell sorter) plots and (**b**) accumulated data from three replicates (mean ± s.d. of geometric mean fluorescence index (GMFI)) of one representative out of three independent experiments are shown. (**c**) Stability of $\alpha_4\beta_7$ expression *in vivo*. CD4$^+$ T_N cells were stimulated two times with plate-bound anti-CD3/CD28 antibodies with or without RA and anti-IL-4 antibody as above. On day 11, cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and transferred into recipient mice. Isolation and analysis were carried out on day 15 after transfer. Mean ± s.d. of pooled GMFI data from two out of three independent experiments with $n = 4$ or 5 mice in total. Student's *t*-test with $^{**}P < 0.01$ and $^{***}P < 0.001$.

Next, we designed amplicons on both regions to analyze the methylation status in CD4$^+$ T cells. As the promoter contained a large CpG-I, which was largely demethylated (data not shown), we concentrated our analysis on two CpG motifs in the CpG-I shore. Shores of CpG-I have been reported to contain most of the dynamic methylation-dependent regulatory capacity, whereas CpG-Is are usually uniformly demethylated in most promoters.[31] Indeed, we found significant demethylation of the two CpG-I shore CpGs in $\alpha_4\beta_7^+$ CD4$^+$ T_{mem} as well as in T cells repeatedly stimulated in the presence of RA (**Figure 5d**). Their $\alpha_4\beta_7^-$ counterparts ($\alpha_4\beta_7^-$ P-lig$^+$ T_{mem}, expressing the inflammation/skin-related homing receptor P-selectin ligand (P-lig), as well as T cells stimulated in the presence of the RA antagonist LE540), displayed high methylation levels. Interestingly, *ex vivo*-isolated T_N cells, which have not yet undergone imprinting for a certain homing phenotype, showed an equally low degree of methylation, suggesting that these cells are permissive for the expression of $\alpha_4\beta_7$ in the presence of appropriate conditions, whereas stimulation in the absence of RA signaling or differentiation into skin-homing T_{mem} promotes acquisition of an inhibitory epigenetic mark at the promoter.

Analysis of seven CpGs in dmr1 showed a significant demethylation in $\alpha_4\beta_7^+$ vs. $\alpha_4\beta_7^-$ CD4$^+$ T_{mem}, whereas RA-induced $\alpha_4\beta_7^+$ T cells displayed only small differences from their counterparts treated with the RA antagonist LE540 or uncommited T_N cells (**Figure 5e**). These data indicate that the methylation level in dmr1 qualifies as a stabilizer for *Itga4* expression in fully imprinted $\alpha_4\beta_7^+$ CD4$^+$ T_{mem}, whereas repetitive stimulation *in vitro* in the presence of RA might not be sufficient to achieve full epigenetic fixation at this regulatory element. As further support for this conclusion, we found increased stability of $\alpha_4\beta_7$ expression in murine T_N, which were treated with the DNA-demethylating drug 5-azacytidine during the RA-mediated induction phase (**Supplementary Figure 4**).

To confirm the putative methylation-dependent enhancer/stabilizer function of dmr1, we cloned the dmr1 into a CpG-free luciferase reporter[32] —which allows selective *in vitro* methylation of inserted CpG-containing sequences— and performed reporter gene assays in primary murine T cells. Indeed, the enhancer activity of dmr1 was strongly compromised upon methylation (**Figure 5c**), supporting a functional link between DNA methylation and transcriptional activity.

The human *ITGA4* gene also shows epigenetic modification in $\alpha_4\beta_7^+$ vs. $\alpha_4\beta_7^-$ CD4$^+$ T cells

The murine *Itga4* and human *ITGA4* loci show a high degree of structural similarity (**Figure 6a**) even if the sequence homology is variable. Therefore, similar regulatory mechanisms might apply. Using whole-genome bisulfite sequencing data of human CD4$^+$ T cells generated in the context of the German Epigenome Programme "DEEP",[33] we analyzed the *ITGA4* promoter and searched for regions that were differentially methylated between naive and memory cells and might correspond to the murine dmr1. Two DMR were identified for which amplicons for the detailed analysis of DNA methylation were designed (**Figure 6a**).

Similar to murine T cells, $\alpha_4\beta_7^+$ CD4$^+$ T cells are found in the memory T-cell fraction in human peripheral blood and $\alpha_4\beta_7$

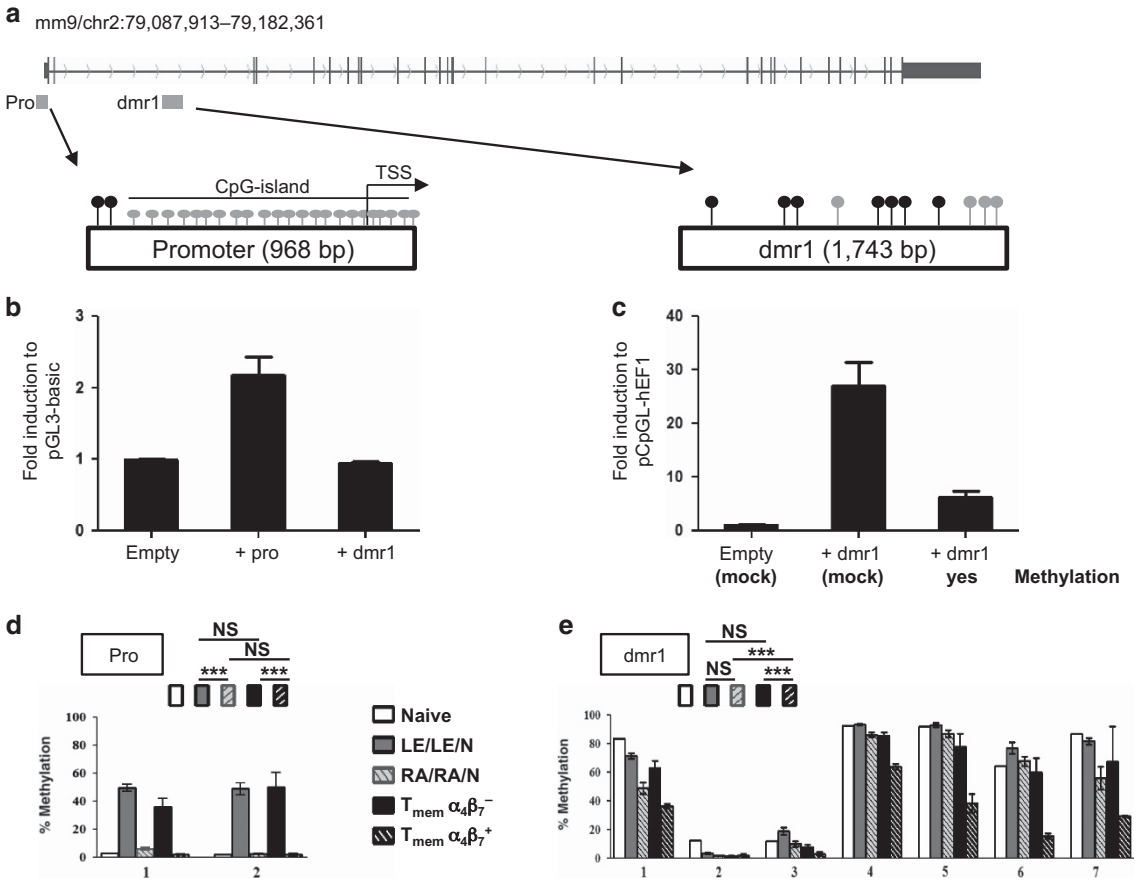

Figure 5 The promoter and an intronic enhancer in the murine *Itga4* locus show transcriptional activity and differential DNA methylation patterns. (**a**) The genomic organization of the murine *Itga4* locus is shown with the position of the analyzed promoter (pro) and enhancer (differentially methylated region 1 (dmr1)) region. The position of CpG motifs within the promoter and in the dmr1 region used in (**b** and **c**) are shown as needle heads. CpG motifs shown in black were analyzed for methylation in (**d** and **e**). (**b** and **c**) The *Itga4* pro and the dmr1 enhancer show promoter or methylation-sensitive enhancer activity, respectively. (**b**) The *Itga4* promoter and the dmr1 region were cloned into the promoter-free pGL3 luciferase reporter plasmids (pGL3-basic) and tested for transcriptional promoter activity in murine $CD4^+$ Th1 cells in a luciferase reporter assay. In addition, dmr1 was tested in a CpG-free luciferase reporter vector (pCpGL-hEF1) containing the hEF1 promoter. This vector allows application of *in vitro* methylation (and mock methylation as control), as this affects only the cloned insert. (**c**) With this approach, the methylation sensitivity of the enhancer function of dmr1 was assessed in primary murine $CD4^+$ T cells. (**d** and **e**) The degree of DNA methylation of selected CpG motifs (shown in **a** as black needle heads) in the (**d**) *Itga4* promoter and (**e**) dmr1 was analyzed by bisulfite sequencing of T_N ($n=2$) and *ex vivo* isolated $\alpha_4\beta_7^+$ or $\alpha_4\beta_7^-$ P-lig$^+$ T_{mem} ($n=3$ each), as well as of cultured T_N after two rounds of retinoic acid (RA) or LE540 treatment followed by a final round in the absence of both additives (N) ($n=3$ each). Mean (±s.d., if $n>2$) methylation values for each CpG motif are given. Statistical significance of differences between all analyzed CpG motifs of the respective cell types in a given region was tested as described in the Method section. Significant differences are indicated: ***$P<0.001$ and NS, not significant. TSS, transcription start site.

expression is mutually exclusive to expression of the skin-homing receptor and E-selectin ligand CLA (**Figure 6b**). In the naive fraction of human $CD4^+$ cells, both $\alpha_4\beta_7^+$ and $\alpha_4\beta_7^-$ cells can be detected using the very bright Act-1-PE antibody (**Figure 6b**), a finding of unknown biological significance that was not reported before and is not observed in murine cells. Compared with T_{mem}, $\alpha_4\beta_7^+$ T_N cells express detectable, but intermediate, levels of $\alpha_4\beta_7$ (**Figure 6b**). We analyzed the degree of DNA methylation for six CpGs in the CpG-I shore of the promoter as well as for DMR1 and DMR2.

Methylation of the analyzed CpGs in the CpG-I shore of the promoter was generally at a low level, but lowest in the $\alpha_4\beta_7$-expressing populations of both T_N and T_{mem} cells (**Figure 6c** and **f**). As observed in murine cells, the highest methylation was found in $\alpha_4\beta_7^-$ (and CLA^+) memory cells, suggesting an epigenetic silencing of *ITGA4* during differentiation into skin-homing T_{mem}. $\alpha_4\beta_7^-$-naive T cells displayed an intermediate methylation state, suggesting that they are predisposed to *ITGA4* expression upon appropriate signaling.

For DMR1, fully commited $\alpha_4\beta_7^+$ CLA^- T_{mem} were the only cell type displaying consistently low levels of methylation among the CpGs of this region, indicating that DMR1 might serve as a stabilizer element (**Figure 6d** and **f**). The $\alpha_4\beta_7^+$ fraction of T_N showed a tendency towards decreased methylation compared with the $\alpha_4\beta_7^-$ cell types, which, however, lacked statistical significance.

Interestingly, the methylation pattern in DMR2 discriminated T_{mem} from T_N, but was not correlated to $\alpha_4\beta_7$ expression (**Figure 6e** and **f**). This suggests that epigenetic opening of this putative regulatory region might already occur with acquisition

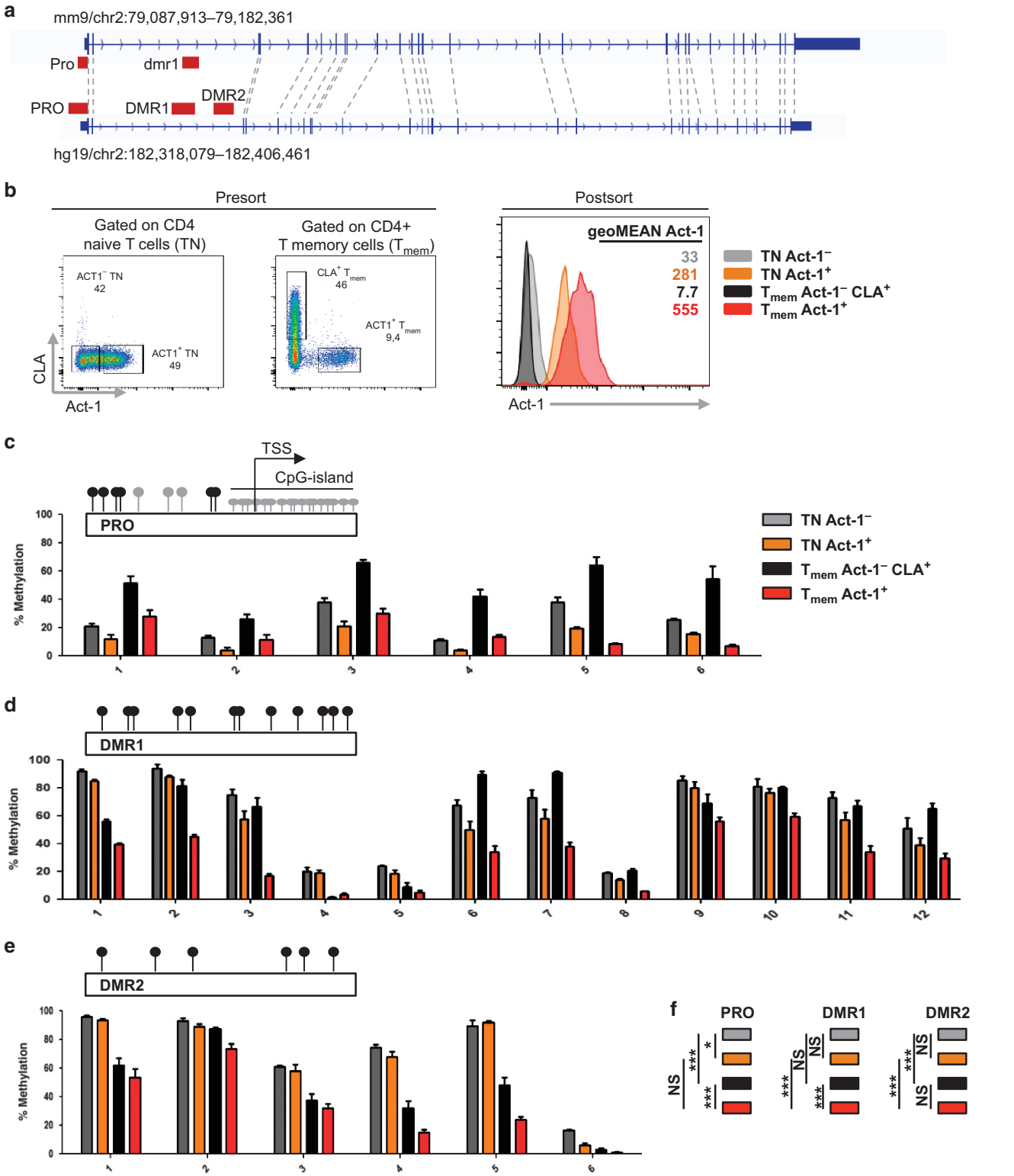

Figure 6 Differential DNA methylation in regulatory regions of the *ITGA4* locus in *ex vivo* human T-cell subsets. (**a**) Structural similarities of the murine *Itga4* (above) and the human *ITGA4* (below) locus are shown as well as the positions of the promoters (pro/PRO) and the analyzed differentially methylated regions (differentially methylated region 1 (dmr1)/DMR1/DMR2). (**b**) Human CD4$^+$ T-cell subsets were sorted according to the gating shown (left). T_N and T_{mem} differ in the level of $\alpha_4\beta_7$ expressed using the Act-1 antibody. (**c**–**f**) The degree of DNA methylation (mean ± s.d., $n = 3$) of selected CpG motifs (shown as black needle heads) in the (**c**) *ITGA4* promoter, (**d**) DMR1 and (**e**) DMR2 were analyzed by bisulfite sequencing in the sorted CD4$^+$ T cell subsets shown in (**b**). (**f**) Statistical analyses of the DNA methylation differences over all CpGs analyzed in the indicated region. Significant differences are indicated: $^*P \leq 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; NS, not significant. TSS, transcription start site.

of a memory state and might allow for expression of *ITGA4* also under conditions, where the α_4-chain, but not the mucosa-specific $\alpha_4\beta_7$-integrin is expressed. This would contrast to the role of dmr1/DMR1, which seems to regulate the α_4-chain only in the mucosa-seeking subset of T_{mem}. In addition, decreased methylation in three regions correlating with upregulated expression in T_{mem} cells was also found for ITGB1, coding for the β1-integrin chain (**Supplementary Figure 6**).

DISCUSSION

The differentiation of T-lymphocytes into memory cells is accompanied by functional specialization, including the acquisition of organ-specific homing capabilities. The present study was designed to answer the question whether, and if so which, cell-intrinsic mechanisms allow T cells primed under appropriate conditions to develop a stable topographical memory for the gut, independent of environmental signals. Our present data demonstrate that indeed an "imprinting for gut homing" is observed for the $\alpha_4\beta_7$-integrin, which requires repetitive instruction by RA signals. In contrast, stable expression is not inducible for CCR9, at least under the conditions tested here. At the molecular level, this imprinting is based on epigenetic mechanisms, notably regulation of DNA (CpG −) methylation at specific regulatory regions of the *Itga4* locus.

The mucosal homing receptor $\alpha_4\beta_7$ integrin is expressed at a low, but functional, level on murine naive lymphocytes and enables their recirculation through gut-associated lymphoid tissues.[34] Upon activation and differentiation into effector/memory cells, the population splits up into T_{mem} that are either completely negative or highly positive for $\alpha_4\beta_7$, the latter being considered as a stable mucosa-specific memory population.[10,35] In contrast, Mora *et al.*[16] studied the expression of $\alpha_4\beta_7$ in $CD8^+$ T cells and reported a high degree of plasticity, as freshly generated $\alpha_4\beta_7^+$ $CD8^+$ T cells could be converted into $\alpha_4\beta_7^-$ cells by restimulation in the presence of subcutanous instead of mucosal DCs, in line with other reports [18]. Moreover, these authors noted that *ex vivo*-isolated $CD8^+$ T_{mem} ($CCR9^+\alpha_4\beta_7^{+/-}CD44^+$) were also found to change their $\alpha_4\beta_7$ expression pattern upon restimulation with the opposite type of DC, suggesting a lack of $\alpha_4\beta_7$ stability in $CD8^+$ T_{mem}. More recently, Masopust *et al.*[17] observed in a comprehensive study a similar instability of $\alpha_4\beta_7$ expression on $CD8^+$ T cells *in vivo*. In addition, they found that $\alpha_4\beta_7$ was efficiently but only transiently upregulated upon *in vivo* activation, regardless of the site of antigen contact.

With these data, the original paradigm of organ-specific homing[1,36] was questioned. We now demonstrate that $CD4^+$ T cells, in contrast, can acquire long-term stability of $\alpha_4\beta_7$ expression and hence mucosa-specific homing, restoring the validity of the homing concept for this subset. In fact, differences in the migratory program of $CD4^+$ and $CD8^+$ T_{mem} including higher stability of selectin ligands on $CD4^+$ T_{mem} have already been reported[15] and are now further corroborated by our present data.

RA, produced from the precursor vitamin A by stromal cells,[11,37] $CD103^+$ DCs[13,38] in gut-associated lymphoid tissues, or by liver sinusoidal cells,[39] is the key inducer of $\alpha_4\beta_7$ by regulating the α_4-chain.[30] In contrast, basic levels of the β_7-chain are constitutively transcribed in $CD4^+$ T cells, outcompeted by high levels of the β_1-chain and regulated by tumor growth factor-β.[30,40] We have shown here that RA signals are sufficient to induce $\alpha_4\beta_7$ expression, but the stability of expression (in the absence of RA) is not immediately achieved. Two rounds of *in vitro* activation in the presence of RA induced a certain degree of stability, yet complete stability with sustained high expression of $\alpha_4\beta_7$ was found only in *ex vivo*-isolated T_{mem}.

Stability *in vivo* may rely on cell-intrinsic mechanisms, but, alternatively, may be caused by continuous exposure to RA according to three different scenarios: (i) exposure to low levels of RA within the circulation;[41] (ii) contact with RA-generating antigen-presenting cells during passage through the liver;[39] (iii) by recirculation through mucosal tissues harboring dendritic cells and/or stromal cells providing RA and/or other unknown instructive signals. However, $\alpha_4\beta_7$ expression was neither lost upon transfer into RA-depleted animals nor reduced when recirculation of transferred cells through mucosal tissues was blocked. Thus, the findings of this study strongly argue for cell-intrinsic mechanisms being responsible for the imprinting of the mucosal homing phenotype.

How could stability be achieved at a molecular level? A growing body of evidence assigns a key role to the epigenetic machinery in the fixation of phenotypic profiles, as shown for various processes of cell differentiation and lineage commitment.[20,33,42] In T cells, effector cytokines, master transcription factors and other genes are subject to epigenetic fixation[21,22] and some indirect hints from our previous work also pointed towards a role in the regulation of homing.[14,43] In particular, DNA methylation at promoters or enhancers appears to be an attractive mechanism for the regulation of cellular memory by control of chromatin accessibility and transcription factor binding, particularly with respect to the heritability of DNA methylation patterns during mitosis.[44]

The results reported here provide first evidence that stable expression of $\alpha_4\beta_7$ in the absence of the initial inducer is indeed due to epigenetic regulation at the level of DNA methylation. In both murine and human $CD4^+$ T cells, the *Itga4/ITGA4* locus was found to display distinct differentially methylated regions (dmr/DMR) whose degree of methylation correlated with $\alpha_4\beta_7$ expression, commitment, and/or memory differentiation.

First, several CpGs upstream of the promoter-associated demethylated CpG-I showed distinct differences in methylation. In murine T_N, which express a low but detectable level of $\alpha_4\beta_7$,[35] the two CpG upstream of the CpG-I were completely demethylated similar to the $\alpha_4\beta_7^+$ fraction of T_{mem}. Although cells repeatedly stimulated in the presence of RA kept this status, stimulation in the absence of RA as well as *in vivo* differentiation into $\alpha_4\beta_7^-$ T_{mem} resulted in a pronounced methylation. Thus, we conclude that the open conformation of this region enables transcription in both T_N and T_{mem}, which, however, seems to be modulated by further regulatory regions, and can be silencend upon differentiation into non-mucosal

subsets of T_{mem}. It has to be mentioned that the degree of methylation was not uniform among the CpGs analyzed. Whether this relates to specific functions of single CpGs, e.g., by being part of a distinct transcription factor-binding motif, remains to be investigated. In human cells, for which an $\alpha_4\beta_7^+$ as well as an $\alpha_4\beta_7^-$ population can be discriminated among T_N, the latter show moderate methylation, whereas the positive fraction is significantly less methylated. Methylation levels in $\alpha_4\beta_7^-$ T_{mem} are much higher than in the $\alpha_4\beta_7^+$ T_{mem} fraction. Thus, the suggested regulatory role of CpG methylation in the promotor-associated region seems to be conserved between mouse and man and to extend to subfractions of naive cells.

Second, differential methylation helped us to identify a novel regulatory region (dmr1) in the murine *Itga4* locus that acts as an enhancer. In contrast to the promotor-associated region, the CpG motifs within dmr1 are almost completely methylated in T_N, and there is only a minor, nonsignificant decrease of methylation upon repeated stimulation in the presence of RA, or in $\alpha_4\beta_7^+$ vs. $\alpha_4\beta_7^-$ T_N. However, the methylation level was strongly reduced in $\alpha_4\beta_7^+$ T_{mem} as compared with $\alpha_4\beta_7^-$ T_{mem} or T_N. The enhancer activity of dmr1 was completely abolished by artificial methylation, supporting the functional relevance of DNA methylation in controlling this region. A further hint at the functional role of DNA methylation on regulation of $\alpha_4\beta_7^+$ expression is the stabilizing effect of 5-azacytidine, which blocks DNA methylation; however, indirect effects of this genome-wide-acting inhibitor cannot be excluded.

A related site designated as DMR1 was identified in human $CD4^+$ T cells, which showed a corresponding demethylation signature correlating to $\alpha_4\beta_7^+$ expression. As an additional indication for a conserved mechanism of regulation via dmr1/DMR1, we found the activating histone modification mark H3K4me1 at the human DMR1, indicating that indeed it may serve as an enhancer element (**Supplementary Figure 5**).

Recently, the transcription factor BATF has been reported to regulate the expression of the α_4-chain in $CD4^+$ cells.[45] According to the ENCODE data,[46] BATF binds in a lymphoid cell line at the promoter region; further binding sites for BATF are predicted at the *ITGA4* locus including at the DMR1 (using tRap and the jaspar database; data not shown), suggesting BATF as one possible transcription factor involved in $\alpha_4\beta_7$ stability.

Whole-genome bisulfite sequencing data of the human *ITGA4* locus uncovered an additional DMR (DMR2). Similar to dmr1/DMR1, CpGs 1–5 were almost completely methylated in both $\alpha_4\beta_7^+$- and $\alpha_4\beta_7^-$-naive cell subsets, but a highly significant drop in methylation was observed in both memory subsets. Here the differences between $\alpha_4\beta_7^+$ and $\alpha_4\beta_7^-$ were less prominent and did not reach statistical significance. Hence, this region might be required for expression of the α_4-chain in T_{mem} in general (e.g. in $\alpha_4\beta_1^+$, but $\alpha_4\beta_7^-$ cells), but additional signals (e.g., from the dmr1/DMR1 enhancer) might regulate it in the mucosa-specific subset.

A subset of effector/memory T cells expresses the related integrin dimer $\alpha_4\beta_1$ ("VLA-4"). $\alpha_4\beta_1$ but not $\alpha_4\beta_7$ has a key role in multiple sclerosis as this integrin is essential for pathogenic T cells to cross the blood–brain barrier[47,48] and can be expressed on effector T cells migrating to further tissues such as the skin and the respiratory track as well. Importantly, expression of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ on memory cells is largely mutually exclusive (data not shown). Our findings on the imprinting of the gut-homing phenotype via epigenetic regulation at DMR1 of the *ITGA4* gene apply to the $\alpha_4\beta_7^+$ subset; $\alpha_4\beta_7^-$ P-lig$^+$ cells, which partially express $\alpha_4\beta_1$, do not show a demethylated DMR1. However, DMR2, which is demethylated in all memory cells could be a candidate for an epigenetically controlled region regulating $\alpha_4\beta_1$ in $\alpha_4\beta_7^-$ T_{mem} cells. Moreover, first evidence that the *ITGB1* locus for his part is also regulated by epigenetic mechanisms can be deduced from the differential methylation in several regions of the human *ITGB1* gene correlating with differential expression in T_N vs. T_{mem} (**Supplementary Figure 6**).

In conclusion, the data of this study confirm the validity of the concept of a topographical memory in $CD4^+$ T_{mem} and provide first evidence that the imprinting of a mucosa-specific homing phenotype involves epigenetic regulation of expression of the α_4-integrin chain.

METHODS

Mice. C57Bl/6 and congenic Thy1.1 C57Bl/6 mice were bred at the Bundesinstitut fuer Risikobewertung (Berlin, Germany) and were used at 8–12 weeks of age. BALB/c mice and 7–9-month-old exbreeders were from Charles River WIGA GmbH (Sulzfeld, Germany). All animal experiments were performed under specific pathogen-free conditions and in accordance with institutional, state, and federal guidelines as approved by Landesamt für Gesundheit und Soziales (LaGeSo) Berlin.

Human blood samples. Buffy coats were from DRK-Blutspendedienst Nord-Ost and were used for isolation of human T-cell populations as approved by the local ethics committee (Ethikkommission, Ethikausschuss 1, CCM; application no. EA1/095/13).

Antibodies and cell culture reagents. Antibodies and fusion proteins used were as follows: anti-mCD4 (RM4-5), anti-mCD25 (PC6.1), anti-mCD45RB (16A), anti-mThy1.1 (OX-7), anti-m$\alpha_4\beta_7$ (DATK32) and streptavidin PE-Cy7 (all BD Pharmingen, Franklin Lakes, NJ), anti-human IgG (Jackson Immuno Research, West Grove, PA), anti-mCCR9 (242503; R&D Systems, Minneapolis, MN), biotinylated anti-m$\alpha_4\beta_7$ (DATK32, anti-mFcγR II/III (2.4G2), anti-mB220 (RA3.6B2), anti-mMac-1 (M1/70.15.11), anti-mCD8 (53-6.7), anti-mCD3 (145.2C11), anti-mCD28 (37.51), anti-OVA-specific mTCR (KJ1.26), anti-mMAdCAM-1 (Meca367), anti-mVCAM-1 (6C7.1), and anti-mIL4 (all produced in house), anti-hCD3 (UCHT1), anti-hCD4 (OKT4), anti-hCD45RO (UCHL1), anti-hCLA (HECA-452) (from Biolegend, San Diego, CA), and anti-hCD45RA (2H4LDH11LDB9; Beckman-Coulter, Brea, CA). Recombinant murine P-selectin-huIgG fusion protein used for labeling P-selectin ligand (P-lig) was kindly provided by D Vestweber (Münster, Germany). The anti-human $\alpha_4\beta_7$ antibody Act-1 was provided by Millenium at Takeda Pharmaceutical Company (Cambridge, MA) and PE-conjugated in-house. Microbeads were from Miltenyi Biotec (Bergisch Gladbach, Germany). Mouse recombinant IL-2 (rmIL-2), rmIL-12, and rmIFNγ (interferon-γ) were from R&D Systems, all-*trans* RA and ovalbumin and cholera toxin were from Sigma-Aldrich, St. Louis, MO, pan-RA receptor antagonist LE540 was from Wako Chemicals (Neuss, Germany). Cell culture was performed in RPMI-1640 (Gibco, Carlsbad, CA and PAA (Cölbe, Germany)) supplemented with 10% fetal calf serum (Sigma).

Flow cytometry. For flow cytometry an LSRII (BD Biosciences, Franklin Lakes, NJ) and FlowJo software were used. Gates were set according to FMO (fluorescence minus one) stainings with or without

isotype controls. Cell sorting was performed on FACSAria I or II Cell Sorter BD, Franklin Lakes, NJ.

Isolation of *ex vivo* murine and human T cells. For *in vitro* culture of murine $CD4^+$ T_N, pooled erythrocyte-depleted spleen and lymph node cells from BALB/c, C57Bl/6, or Thy1.1 congenic C57Bl/6 mice were stained with anti-mCD4-FITC and anti-mCD25-APC antibodies and depleted of $CD25^+$ cells using anti-APC microbeads and the AutoMACS magnetic separation system (Miltenyi Biotec). Subsequently, $CD25^-$ cells were positively enriched for $CD4^+$ T cells using anti-FITC multisort beads. Release reagent and anti-CD62L microbeads were used to obtain $CD25^-CD4^+CD62L^{high}$ T cells.

For the isolation of murine $CD4^+$ T_{mem}, pooled spleen, peripheral, and mesenteric lymph node cells from 20 to 40 BALB/c exbreeder mice were negatively selected with anti-mCD8, anti-mMac-1, and anti-mB220 antibodies and anti-rat-IgG microbeads. Enriched $CD4^+$ T cells were labeled with anti-CD45RB FITC and depleted of $CD45RB^{hi}$ cells using anti-FITC microbeads. Subsequently, $\alpha_4\beta_7^+$ P-lig$^-$ $CD45RB^{low}CD4^+$ and $\alpha_4\beta_7^-$ P-lig$^-$ $CD45RB^{low}CD4^+$ T cells were sorted by flow cytometry. For methylation analyses, the $\alpha_4\beta_7^-$ P-lig$^+$ subset ("inflammation/skin-specific subset") was sorted as the $\alpha_4\beta_7^-$ subset.

For isolation of human T cells, peripheral blood mononuclear cells were isolated by density gradient centrifugation using Lymphocyte Separation Medium LSM 1077 (PAA, Cölbe, Germany) from buffy coats. Erythrocytes were lysed in erythrocyte lysis buffer (Buffer EL; Qiagen, Venlo, Niederlande). $CD4^+$ T-lymphocytes were enriched using CD4 MicroBeads (Miltenyi Biotec). The enriched population was stained and sorted by flow cytometry into four populations:
T_N Act-1$^-$ = $CD3^+CD4^+CD45RA^+CD45RO^-$Act-1$^-CLA^-$;
T_N Act-1$^+$ = $CD3^+CD4^+CD45RA^+CD45RO^-$Act-1$^+CLA^-$;
T_{mem} Act-1$^-$ CLA$^+$ = $CD3^+CD4^+CD45RA^-CD45RO^+$Act-1$^-CLA^+$;
T_{mem} Act-1$^+$ = $CD3^+CD4^+CD45RA^-CD45RO^+$Act-1$^+CLA^-$.

Purity of the sorted populations was confirmed by flow cytometry and >95%.

***In vitro* cell cultures**. Murine naive $CD25^-CD4^+CD62L^{high}$ T cells were stimulated on plate-bound anti-mCD3 (1–4 μg ml^{-1}) and anti-mCD28 (4–8 μg ml^{-1}) antibodies or via irradiated APCs with soluble anti-mCD3 and anti-mCD28 in the presence of rmIL-2 (10 ng ml^{-1}) either under neutral (no further supplements) or under Th1 polarizing conditions (rmIL-12 (5 ng ml^{-1}), rmIFNγ (20 ng ml^{-1}), anti-mIL-4 (5 μg ml^{-1})), with or without the addition of RA (1 nM) or LE540 (0.25 μM, to block medium-derived RA). On day 3 of culture, T cells were removed from the stimulus and rested for another 1–3 days. Cells were analyzed for $\alpha_4\beta_7$ expression on the indicated days and restimulated for prolonged culture periods on days 5 and 11. In contrast to T_N, *ex vivo* isolated T_{mem} were stimulated with reduced levels of plate-bound anti-mCD3 (1 μg ml^{-1}) and anti-mCD28 (2 μg ml^{-1}) for 12–16 h and subsequently rested until restimulation.

Analysis of homing receptor expression on adoptively transferred T cells. A total of 1.5–3 × 10^6 cultured $CD4^+$ T cells (day 11 of culture) or *ex vivo* isolated, CFSE-labeled or Thy1.1/2 congenic T_{mem} were injected into the tail vein of recipients. On day 15 or 6 weeks after T-cell transfer, recipient mice were killed and cells from spleen, pLN, and mLNs were stained for CD4, Thy1.1, and $\alpha_4\beta_7$. At these time points, between 0.12 and 0.05% of the $CD4^+$ T cells in lymphoid organs were donor-derived.

Generation of vitamin A-deficient mice. Female mice received a vitamin A-deficient (ssniff Spezialdiäten GmbH, Soest, Germany) or control diet 10 days after initial mating (embryonic days 7–10). Offspring was weaned at 3 weeks of age and on diet until 19 weeks of age before transfer of T_{mem}. To verify depletion of vitamin A, CFSE-labeled OVA-specific $CD4^+CD62L^{high}$ T cells from DO11.10 mice were transferred to mice fed vitamin A-reduced or control diet. On day 1 after transfer, mice were immunized with 100 mg OVA protein and 10 μg cholera toxin (both Sigma-Aldrich) by oral gavage. Four days after immunization, $\alpha_4\beta_7$ and CCR9 expression were analyzed on OVA-specific (KJ^+) mLN cells.

Exclusion of $\alpha_4\beta_7^+$ T_{mem} from GALT. To exclude $\alpha_4\beta_7^+$ T_{mem} from GALT, recipient mice received either rat IgG or anti-MAdCAM-1 (Meca367; 500 μg per mouse) plus anti-VCAM-1 (6C7.1; 200 μg per mouse) antibodies intravenously every 3 days. This treatment started on the day of transfer and was maintained until the analysis of $\alpha_4\beta_7$ expression on day 15.

Generation of luciferase reporter vectors. The *Itgα4* inserts were amplified by PCR using mouse genomic DNA and the following primers (Eurofins, Hamburg, Germany): *Itga4* promoter_fwd: 5′-CTG GTGGTAGGTATGTCCTGGGGT-3′ and *Itga4* promoter_rev: 5′-CG CTCTTGGTGGAGAACATT-3′; dmr1_fwd: 5′-TCAGATTTTGCTA GCCATCCT-3′ and dmr1_rev: 5′-TGCTTCCCACAATTCTAAAA CA-3′. PCR fragments for the *Itga4* promoter and dmr1 were cloned into the pGL3-basic luciferase reporter vector (Promega, Madison, WI, USA). For the generation of a reporter vector for dmr1 in a CpG-free background,[32] the dmr1 region was inserted into the pCpGL-EF1 vector.[49] Endotoxin-free plasmid DNA for transfection was purified using NucleoBond Xtra Maxi EF (Macherey-Nagel, Düren, Germany).

Luciferase reporter assay. Murine-naive $CD25^-CD4^+CD62L^{high}$ T cells were stimulated for two days using plate-bound antibodies and polarized towards Th1 as described above. A total of 1 × 10^6 *in vitro*-induced C57Bl/6 Th1 cells were transfected with equimolar amounts (3.12 pmol) of the reporter plasmids using the Neon transfection device (Life Technologies, Carlsbad, CA) applying two pulses (voltage: 1,350 V; width: 20 ms). Fifty nanograms of the pRL-CMV vector (Promega) containing the Renilla luciferase cDNA were co-transfected and used as an internal control for transfection efficiency. Cells were cultured for another 24 h with and another 24 h without plate-bound stimulation antibodies. Cells were harvested and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). After normalization of firefly activity to Renilla activity, relative firefly activity was normalized to activity of the empty vector.

***In vitro* methylation of luciferase reporter plasmids**. Thirty micrograms of plasmid DNA were incubated with M.*Sss*I (2.5 U μg^{-1} DNA) in the presence of 160 μM *S*-adenosylmethionine for 4 h at 37 °C, with another 160 μM *S*-adenosylmethionine added after the first 2 h of incubation. Mock-methylated plasmids were treated identically but without the enzyme and *S*-adenosylmethionine. Plasmid DNA was purified using the NucleoSpinExtract II Kit (Macherey-Nagel). Successful methylation was verified with the methylation-sensitive restriction enzyme *Hpa*II.

DNA methylation analysis by bisulfite sequencing. Bisulfite sequencing for murine and human samples was performed according to Gries *et al.*[50] (primers in **Supplementary Table 1**). Briefly, 300 ng genomic DNA was bisulfite treated using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). PCRs were performed using primers with a specific 3′-portion and a universal 5′-portion according to Illumina's specifications (specific portion of primer sequences are listed in **Supplementary Table 1**). Amplicons were purified using AGENCOURT Ampurebeads (Beckman/Coulter), diluted, and pooled. Deep sequencing was performed on the Illumina (San Diego, CA) MiSeq according to the manufacturer's protocols aiming at 10,000 reads per amplicon. Reads were processed and aligned using the BiQ Analyzer HT software (http://www.mpi-inf.mpg.de)[51] setting the maximal fraction of unrecognized sites' filter at 0.1.

Statistics. If not indicated otherwise, data are presented as mean ± s.d. or mean ± s.e.m.). Significance was determined with Student's *t*-test, after testing of normal distribution by the Kolmogorov–Smirnov test. Differences were considered statistically significant with $^*P \leq 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ Differences in DNA methylation were

analyzed by computing differences of permuted methylation levels on each CpG site. Statistical significance was determined by applying one-sample, one-tailed Student's *t*-test on the differences in all CpG in the respective region, with the alternative hypothesis of true methylation difference assumed as $>10\%$ or $<-10\%$. Significance levels ($^{*}P \leq 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$) were in accordance with the nonparametric, one-sample Wilcoxon's signed-rank test with similar settings.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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AUTHOR CONTRIBUTIONS

B.S., J.T., M.M., and A.S. performed functional T-cell analyses; J.T., C.K., M.d.A., M.M., and S.F. were involved in epigenetic analyses; S.T. generated bisulfite-seq data, P.D. performed bioinformatic analyses, A.H. and J.K.P. designed and coordinated the study; B.S., A.H., and J.K.P. wrote the manuscript with contributions from JW, US, and other authors.

DISCLOSURE

The authors declared no conflict of interest.

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