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Complementary roles of retinoic acid and TGF-β1 in coordinated expression of mucosal integrins by T cells

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α_4 and β_7 integrins, such as $\alpha_4\beta_1$, $\alpha_4\beta_7$, and $\alpha_E\beta_7$, are major integrins required for migration of leukocytes into mucosal tissues. The mechanisms responsible for coordinated expression of these three integrins have been poorly elucidated to date. We report that expression of the Itg-α_4 subunit by both CD4$^+$ and CD8$^+$ T cells requires the retinoic acid signal. In contrast, transcription of Itg-α_E genes is induced by the transforming growth factor-β1 (TGFβ1) signal. Expression of Itg-β_7 is constitutive but can be further increased by TGFβ1. Consistently, expression of α_4-containing integrins is severely suppressed in vitamin A deficiency with a compensatory increase of $\alpha_E\beta_7$, whereas expression of Itg-α_E and Itg-β_7 is decreased in TGFβ-signal deficiency with a compensatory increase in $\alpha_4\beta_1$. The retinoic acid-mediated regulation of α_4 integrins is required for specific migration of T cells *in vitro* and *in vivo*. These results provide central regulatory mechanisms for coordinated expression of the major mucosal integrins.

INTRODUCTION

Integrin α_4, a subunit of $\alpha_4\beta_1$ (CD49d–CD29) and $\alpha_4\beta_7$, is a major target of intervention in treating inflammatory diseases through blocking leukocyte migration.[1,2] $\alpha_4\beta_1$, also known as VLA4 (very late antigen 4), is expressed by T cells, B cells, monocytes, and eosinophils.[3,4] $\alpha_4\beta_7$, once called lamina propria-associated molecule 1, is expressed by T cells and B cells in mucosal tissues.[5,6] $\alpha_4\beta_1$ and $\alpha_4\beta_7$ each bind its major counter receptor vascular cell adhesion molecule 1 (VCAM-1) and Mad CAM-1, respectively. $\alpha_4\beta_7$ also binds VCAM-1 and fibronectin, but at reduced affinity.[7] The α_4 integrins are involved in both rolling and firm adhesion of leukocytes on endothelial cells.[8,9] $\alpha_4\beta_1$ is involved in leukocyte migration to diverse tissues including mucosal tissues, bone marrow, splenic follicles, and inflamed tissues, whereas $\alpha_4\beta_7$ has a more specific role in lymphocyte migration to the gut and associated lymphoid tissues.[10–14] $\alpha_E\beta_7$, an integrin related to $\alpha_4\beta_1$ and $\alpha_4\beta_7$, binds E-cadherin expressed on epithelial cells.[15] $\alpha_E\beta_7$ is involved in the localization and function of both effector and regulatory T cells.[16,17]

Retinoic acid, such as all-*trans* retinoic acid (RA), is a vitamin A metabolite and highly produced by epithelial cells and dendritic cells in the small intestine.[18] RA has a number of regulatory functions in the immune system. It is required for differentiation of promyelocytes into neutrophils.[19–21] It promotes the generation of small intestine-homing T and B cells.[22,23] In this regard, a severe paucity of T cells and immunoglobulin A-producing B cells occurs in the intestine because of vitamin A deficiency. These functions of RA are consistent with the increased susceptibility of vitamin A-deficient subjects to a number of infectious microbial agents.[24] RA specifically induces the expression of a small intestine-homing chemokine receptor, chemokine (C-C motif) receptor 9 (CCR9), and a mucosal tissue-homing integrin molecule $\alpha_4\beta_7$.[22]

It has been unclear how the mucosal integrins such as $\alpha_4\beta_1$, $\alpha_4\beta_7$, and $\alpha_E\beta_7$ are coordinately expressed. Through genome-wide gene expression and functional studies, we identified that expression of the Itg-α_4 chain in T cells requires RA, whereas optimal transcription of *Itg*-α_E and to a lesser degree *Itg*-β_7 genes requires the transforming growth factor-β (TGFβ) signal. We provide detailed evidence that functional expression of Itg-α_4- or Itg-α_E-subunit-containing integrins by T cells is coordinately regulated by RA and TGFβ1. The effect of the integrin regulation pathways on T-cell migration in physiological settings is discussed.

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RESULTS

RA selectively induces the transcription of *Itg*-α_4 gene

To understand the function of RA in regulation of mucosal integrin expression in T cells, we activated naive $CD4^+$ T cells in the presence of exogenous RA at a physiologically relevant concentration (10 nM). As controls, we cultured the T cells without exogenous RA or in the presence of Ro41-5253 (a retinoic acid receptor-α (RARα) antagonist; hereafter simply referred to as "Ro41") to block the effect of residual RA that is present in normal culture medium supplemented with 10% fetal bovine serum. It is estimated that 0.2–0.5 nM of RA is present in the culture medium because animal plasma–serum typically contains RA at 2–5 nM.[25] We performed a genome-wide microarray study and found that the *Itg*-α_4 gene is highly induced by the serum-derived RA present in the culture medium at low concentrations (**Figure 1a**). Interestingly, the microarray data revealed that expression of Itg-β_1, Itg-β_7, and Itg-α_E was not significantly affected by different levels of RA (**Figure 1b**). We followed up the data with a real-time PCR assay and found that the *Itg*-α_4 gene is responsive to RA in transcription, whereas the expression of *Itg*-β_1 and *Itg*-β_7 was not significantly affected by the presence or absence of the RA signal (**Figure 1c**). Itg-α_4 transcription was further increased by exogenous RA at 1 and 10 nM. Interestingly, the real-time PCR assay revealed that Itg-α_E mRNA was upregulated when Ro41 was used, which the microarray study failed to reveal.

We performed a chromatin immunoprecipitation assay to assess the potential binding of nuclear RARα to the regulatory region of the *Itg*-α_4 gene. On the basis of the DNA sequence analysis, we found a total of seven putative retinoic acid response elements (RAREs). The chromatin immunoprecipitation assay revealed that one of the putative RAREs is a real binding site for RARα (**Figure 1d**). RA enhanced the binding whereas Ro41 decreased the binding.

In addition to *Itg*-α_4, many genes are up- or downregulated in T cells in response to RA. These genes are listed in **Table 1** and in **Supplementary Figure S1** online. Induction of CCR9 by RA, as reported previously,[22] was clearly detectable. We confirmed by a real-time PCR method that growth hormone-regulated TBC protein 1 (GRTP1), cytochrome *P*450, family 26, subfamily b, polypeptide 1 (CYP26B1), dehydrogenase–reductase member 3 (DHRS3), and leucine zipper transcription factor-like 1 (LZTFL1) are induced by RA, whereas serine (or cysteine) proteinase inhibitor clade B member 1a (SERPINB1) is suppressed by RA (**Supplementary Figure S1** online). GRTP1 is a GTPase activating protein that increases GTPase activity of Rab3A.[26] CYP26B1 is a cytochrome *P*450 that catabolizes RA.[27] DHRS3 is an enzyme that mediates the first oxidative conversion of retinol into retinal.[28] LZTFL1 is a putative transcription factor with a leucine zipper domain and is a part of a transcriptional map that includes the *CCR9* gene.[29] SERPINB1 is an inhibitor of serine proteases such as elastase, cathepsin G, and proteinase-3.[30] We focused our study in this report on regulation of the major mucosal integrins including Itg-α_4.

T-cell activation in the presence of RA induces surface expression of both $\alpha_4\beta_1$ and $\alpha_4\beta_7$

Surface expression of integrins involves heterodimerization of integrin α and β subunits, and therefore simple expression of one subunit is not sufficient for its expression on the cell surface. It is a question of interest whether the α_4 subunit induced by RA is required for all α_4 subunit-containing integrins. We examined whether RA would have significant effects on surface expression of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ (**Figure 2a**). T cells activated in the presence of Ro41 lost expression of Itg-α_4. In contrast, Itg-β_1 was expressed at high levels even with Ro41. Surface expression of Itg-β_7 was enhanced with RA. Induction of surface Itg-α_4 expression by RA is independent of exogenous TGFβ1 in culture. Itg-α_E expression was not significantly changed by RA alone but increased with exogenous TGFβ1. These results, together with the regulation at the RNA level (**Figure 1c**), suggest that RA-dependent induction of *Itg*-α_4 gene transcription is a driving force in the surface expression of both Itg-β_7 and Itg-β_1.

We also assessed the expression of the $\alpha_4\beta_7$ heterodimer complex with the DATK32 antibody (**Figure 2b**). By gating out the $\alpha_4\beta_7^+$ cells, expression of the $\alpha_4\beta_1$ complex by $\alpha_4\beta_7^-$ T cells was also determined. It was apparent that expression of both $\alpha_4\beta_7$ and $\alpha_4\beta_1$ was increased in response to the RA signal.

To gain more insights into the RAR receptor usage, we used additional RAR agonists and antagonists such as LE540 (pan-RAR antagonist), CD2665 (RAR-β/γ antagonist), AM580 (RARα agonist), and AC55649 (RARβ2 agonist) along with RA (pan-RAR agonist) and Ro41 (RARα antagonist; **Figure 2c**). Although both AM580 and AC55649 induced the expression of Itg-α_4, AM580 was more potent even at a lower dose (20 nM). Ro41 was more potent than CD2665. LE540 was most potent in the induction of Itg-β_7 and Itg-α_E. Overall, this information suggests that both the RARα and RARβ/γ receptors can regulate expression of the integrins, but RARα seems to have a larger role.

Flow cytometric determination of the perfect coexpression of Itg-α_E and Itg-β_7 or Itg-α_4 and Itg-β_7 revealed sharp needle-like double-positive populations that may be viewed as the result of autofluorescence or miscompensation in flow cytometry. Using appropriate isotype controls, we confirmed that these double-positive cells indeed have perfect coexpression of integrin subunits (**Supplementary Figure S2** online).

Regular fetal bovine sera contain biologically active RA. We performed a similar culture experiment in a serum-free medium to rule out the effect of the residual RA (**Supplementary Figure S3** online). We confirmed that RA induces Itg-α_4. The T cells of the control group expressed Itg-α_4 at levels similar to those of the Ro41-treated group. This rules out the possibility that the decreased Itg-α_4 expression by Ro41 in a regular medium is because of an unexpected agonistic effect of Ro41.

We further examined the stability of the expressed integrins induced by RA using cycloheximide, a protein biosynthesis inhibitor (**Supplementary Figure S4** online). We found that the induced integrins ($\alpha_4\beta_7$, Itg-α_4, Itg-β_7, Itg-β_1, and Itg-α_E) were stable on the cell surface for at least 12 h after the treatment of cycloheximide.

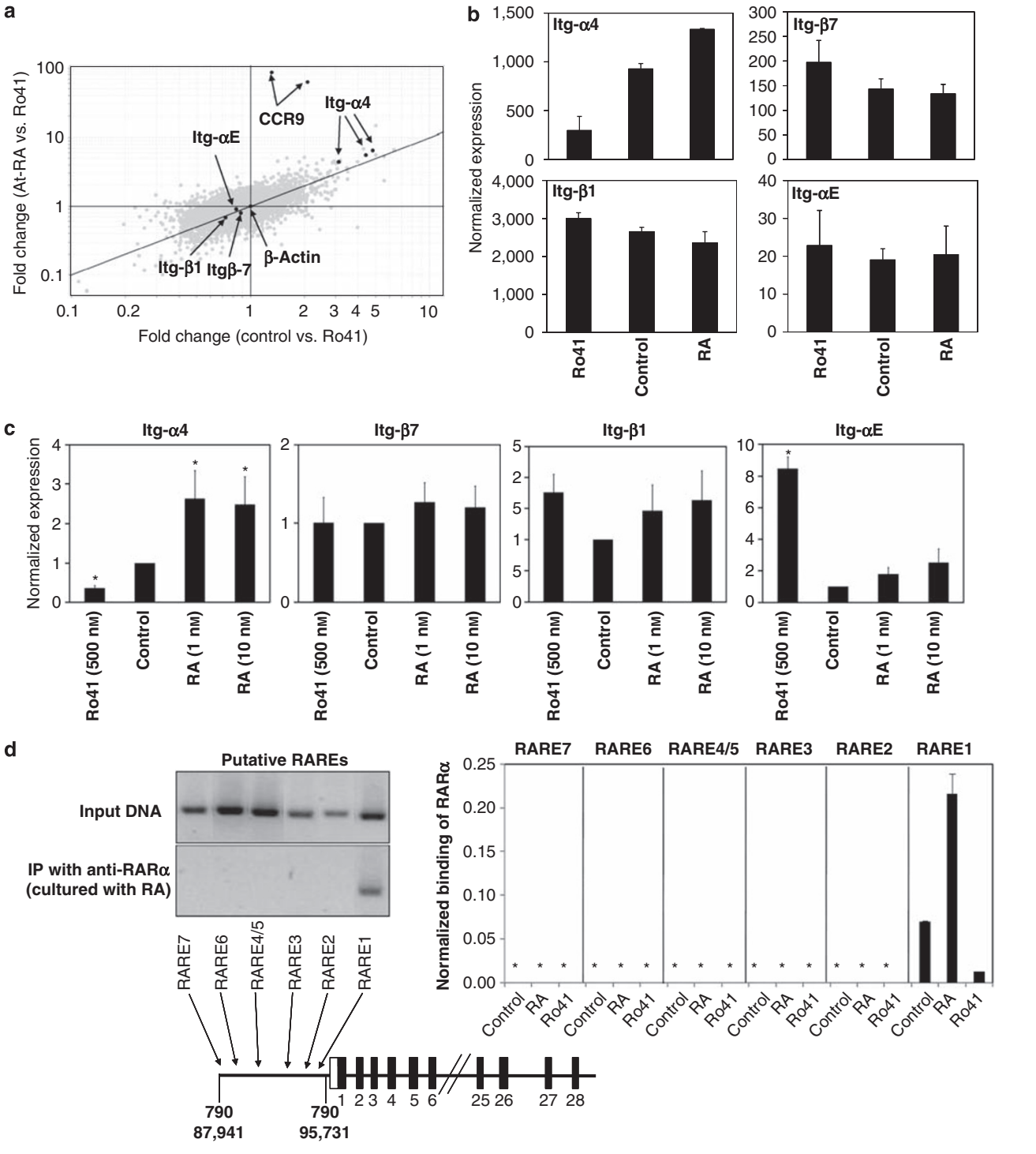


Figure 1 Retinoic acid (RA)- and retinoic acid receptor-α (RARα)-dependent transcription of the *Itg-*α_4 gene, but not *Itg-*β_7, *Itg-*β_1, and *Itg-*α_E genes in CD4$^+$ T cells. (**a**) A dot plot showing genes upregulated in response to high and low concentrations of RA. An Affymetrix microarray was used to determine the levels of gene expression. The *x* axis represents fold changes in gene expression between CD4$^+$ T cells activated in control medium (containing low levels of RA) and CD4$^+$ T cells cultured in the presence a RARα antagonist (Ro41-5253, abbreviated as "Ro41"). The *y* axis represents fold changes in gene expression between CD4$^+$ T cells activated with exogenous RA (10 nM) and CD4$^+$ T cells cultured in the presence a RARα antagonist (Ro41-5253). (**b**) Expression levels of integrin genes based on the microarray data. Error bars are differences between two independent array data sets. (**c**) Real-time PCR analysis of gene expression. Combined data of four independent sets are shown. The data are expressed relative to control T cells. *Significant differences from the controls. (**d**) Binding of RARα to retinoic acid response elements (RAREs) in the 5' upstream regulatory region of the *Itg-*α_4 gene. A chromatin immunoprecipitation (ChIP) assay was performed to determine RARα binding to RARE candidates on the 5' upstream regulatory region of the *Itg-*α_4 gene. RA (10 nM) was used. A representative data set from three independent experiments is shown. *Undetectable.

Table 1 T-cell genes regulated by RA at different RA conditions

Name	Affymetrix ID	GenBank ID	RA/Ro	Con/Ro	RA/Con
RA upregulated genes					
Ccr9	1427419_x_at	NM_009913	86.85	1.31	66.08
Ccr9	1421920_a_at	NM_009913	63.05	2.09	30.14
Cyp26b1	1460011_at	NM_175475	38.08	1.36	27.87
1810011H11Rik	1429604_at	NM_001163616	22.39	10.64	2.10
Grtp1	1425891_a_at	NM_025768	17.11	27.07	2.07
Grtp1	1439150_x_at	NM_025768	14.57	5.03	2.89
P2rx7	1419853_a_at	NM_0010388397	11.92	10.61	1.12
P2rx7	1439787_at	NM_001038839	10.70	8.48	1.26
Dhrs3	1448390_a_at	NM_011303	6.87	4.27	1.60
Osgin1	1424022_at	NM_027950	6.56	6.31	1.04
Laptm5	1459841_x_at	NM_010686	6.49	3.19	2.03
Cerkl///Itga4	1456498_at	NM_010576	6.48	4.82	1.34
Fam102b	1455033_at	NM_001163567	5.53	5.00	1.10
Cerkl///Itga4	1450155_at	NM_010576	5.49	4.41	1.24
Fam102b	1434828_at	NM_001163567	5.30	5.58	0.94
Nrp1	1448943_at	NM_008737	4.81	2.52	1.90
Art2b	1420794_at	NM_019915	4.70	3.15	1.49
Adam19	1418403_at	NM_009616	4.59	2.93	1.56
Lztfl1	1417170_at	NM_033322	4.47	3.97	1.12
Nt5e	1428547_at	NM_011851	4.47	3.97	1.12
Cerkl///Itga4	1436037_at	NM_010576	4.44	3.09	1.43
Adam19	1418402_at	NM_009616	4.35	2.88	1.51
Sorcs2	1419358_at	NM_030889	4.34	2.86	1.51
Cd38	1433741_at	NM_007646	4.32	3.02	1.42
Trim16	1452362_at	NM_053169	4.18	2.33	1.79
Nrgn	1423231_at	NM_022029	4.16	3.02	1.37
Hic1	1449226_at	NM_001098203	3.60	3.47	1.03
Siglec5	1424975_at	NM_145581	3.48	3.06	1.13
Tnfsf11	1419083_at	NM_011613	3.28	2.79	1.17
Pank3	1426259_at	NM_145962	3.15	3.49	0.90
Gm13305	1459868_x_at	NM_001099348	3.11	2.70	1.15
Golga1	1432054_at	NM_029793	2.92	2.69	1.08
Cldn10	1426147_s_at	NM_001160096	2.76	2.70	1.02
Stk17b	1430165_at	NM_133810	2.69	2.88	0.93
Pvt1	1450541_at	NR_003368	2.62	2.56	1.02
Myo1e	1428509_at	NM_181072	2.60	3.08	0.84
D5Wsu178e	1442069_at	NM_027652	2.47	3.48	0.71
Prg2	1422873_at	NM_008920	2.36	5.57	0.42
Gucy1a3	1420533_at	NM_021896	2.23	2.64	0.84
RA downregulated genes					
Tph1	1419524_at	NM_001136084	0.05	0.12	0.48
Cma1	1449456_a_at	NM_010780	0.07	0.11	0.71
Serpinb1a	1416318_at	NM_025429	0.10	0.32	0.31
Nacc2	1417153_at	NM_001037098	0.11	0.25	0.45
1110001D15Rik	1429582_at	NM_001037098	0.13	0.29	0.46

Table 1 Continued on the following page

Table 1 Continued

Name	Affymetrix ID	GenBank ID	RA/Ro	Con/Ro	RA/Con
Mpeg1	1427076_at	NM_010821	0.13	0.45	0.31
Nacc2	1417152_at	NM_001037098	0.14	0.22	0.62
Ifit3	1449025_at	NM_010501	0.15	0.33	0.45
Rsad2	1421009_at	NM_021384	0.15	0.51	0.30
Ifi44	1423555_a_at	NM_133871	0.15	0.36	0.43
Serpinb1a	1448301_s_at	NM_025429	0.16	0.31	0.53
Oasl2	1453196_a_at	NM_011854	0.17	0.41	0.41
Scin	1450276_a_at	NM_001146196	0.18	0.23	0.79
Ccr5	1424727_at	NM_009917	0.19	0.31	0.60
Clec4e	1420330_at	NM_019948	0.21	0.38	0.55
Ctsg	1419594_at	NM_007800	0.22	0.40	0.56
Il1841	1421628_at	NM_001161842	0.28	0.23	1.20
App	1427442_a_at	NM_007471	0.35	0.34	1.02

Naive $CD4^+$ T cells were cultured in three different conditions containing 10 nM retinoic acid (RA), no exogenous RA (Con; low levels of residual RA present in the medium containing 10% fetal bovine serum (FBS)), and Ro41 (Ro; a retinoic acid receptor-α (RARα) antagonist to block the effect of residual RA on RARα). Expression ratios of genes that are up- or downregulated in three different comparisons are shown. The raw and processed data are deposited at the Gene Expression Omnibus (GEO) array data base (www.ncbi.nlm.nih.gov/geo; the accession number is GSE20500).

T-cell expression of Itg-α_4 is decreased in vitamin A deficiency

To confirm the regulatory role of RA *in vivo*, we induced vitamin A deficiency in mice and determined the expression of the integrins. We examined the integrin expression phenotype of T cells in the small intestine and spleen (a non-intestinal tissue). In the spleen, Itg-α_4 was greatly decreased on both $FoxP3^-$ and $FoxP3^+$ $CD4^+$ T cells in vitamin A-deficient mice compared with control mice (**Figure 3a**). This decrease was relatively more severe on $FoxP3^+$ T cells compared with $FoxP3^-$ T cells. Itg-$\beta_1$expression was not affected by different vitamin A status. Itg-β_7 was, unexpectedly, induced in vitamin A deficiency. In the small intestine, Itg-α_4 was again decreased in vitamin A deficiency with almost no or minor change in the expression of Itg-β_1 (**Figure 3b**). Itg-β_7 was again strongly induced in vitamin A deficiency in the small intestine.

Increased surface expression of Itg-β_7 in vitamin A deficiency is a mystery given the current perception that RA would induce Itg-β_7 expression. We hypothesize that the enhanced expression of Itg-β_7 in vitamin A deficiency would be the result of an increased expression of it's other dimerization partner, Itg-α_E, in vitamin A deficiency. When we examined the expression of Itg-α_E and Itg-β_7, it was clear that most Itg-β_7 molecules were coexpressed on T cells with the Itg-α_E molecules in vitamin A deficiency (**Figure 3c, d**). This coexpression was evident on T cells in both spleen and small intestine. The tight coexpression of Itg-α_E and Itg-β_7 suggests that two subunits are probably complexed together in the same cells. Again, this regulation in vitamin A deficiency occurs on both $FoxP3^+$ and $FoxP3^-$ T cells. Overall, there is a clear change from T cells expressing $\alpha_4\beta_1$ and $\alpha_4\beta_7$ in the mice with normal vitamin A status to those expressing $\alpha_E\beta_7$ in vitamin A deficiency (**Supplementary Figure S5** online).

The TGFβ signal is required for functional expression of Itg-β_7 and Itg-α_E

A question critical for expression of both $\alpha_4\beta_7$ and $\alpha_E\beta_7$ is what would regulate the transcription of the *Itg*-β_7 gene. TGFβ is implicated in upregulation of Itg-β_7 in a cytotoxic lymphoma cell line.[31] We examined whether the TGFβ signal is required for expression of Itg-β_7 in primary $CD4^+$ T cells using T cells isolated from transgenic mice expressing a dominant negative form of TGFβRII (dnTGFβRII mice).[32] The T cells in these mice are largely defective in reception of the TGFβ signal. Naive T cells from the dnTGFβRII mice were ineffective in surface expression of Itg-β_7 in response to RA, whereas expression of Itg-α_4 was induced normally (**Figure 4a**). The induction of Itg-β_7 on wild-type $FoxP3^+$ T cells was even higher, whereas it was defective on the transgenic $FoxP3^+$ T cells in the presence of exogenous TGFβ1. In addition, we observed that the surface expression of Itg-α_E and Itg-β_7 was induced in response to TGFβ1 and Ro41 on wild-type but not on the transgenic T cells (**Figure 4a**). CD103–αEβ7, induced by TGFβ1 as determined in this study, is commonly viewed as a $FoxP3^+$ T cell-specific marker. We would like to point out that this is not accurate, because the majority of $CD4^+$ $CD103^+$ T cells in non-lymphoid tissues such as the lung and intestine are $FoxP3^-$ T cells (**Supplementary Figure S6** online). Moreover, even $CD4^+$ $FoxP3^-$ T cells, differentiated *in vitro* in the presence of TGFβ1, highly expressed CD103 (**Figure 4a**).

We next determined the expression levels of mRNA for Itg-α_E and Itg-β_7 in the wild-type and dnTGFβRII T cells cultured with RA or Ro41. We observed that dnTGFβRII T cells fail to express Itg-α_E and Itg-β_7 transcripts (**Figure 4b**). These results show that the TGFβ1 signal is required for expression of Itg-β_7 and Itg-α_E at the RNA level.

To gain insights into the function of the TGFβ signal *in vivo*, we determined the integrin expression phenotype of the T cells from dnTGFβRII mice. We found that expression of Itg-α_E and Itg-β_7 was decreased on CD4$^+$ T cells in the spleen, small intestine, and large intestine (**Figure 4c**). Instead, there were increases in T cells expressing $\alpha_4\beta_1$, which seems to be a compensatory response to the Itg-β_7 decrease (**Figure 4d**). CD8$^+$ T cells were highly similar to CD4$^+$ T cells in expression of the integrins in dnTGFβRII mice.

Regulation of the integrins in CD8$^+$ T cells

The results in **Figure 4c, d** on CD8$^+$ T cells show that optimal expression of $\alpha_E\beta_7$ requires the TGFβ signal. We determined further whether expression of Itg-α_4 and other Itg chains is induced by RA in CD8$^+$ T cells. Similar to CD4$^+$ T cells, Itg-α_4 was strongly induced in response to RA (**Figure 5a**). Expression of Itg-α_E and Itg-β_7 was induced in response to TGFβ1. The overall Itg expression pattern of the CD8$^+$ T cells cultured in a serum-free medium was similar to that cultured in a serum-containing

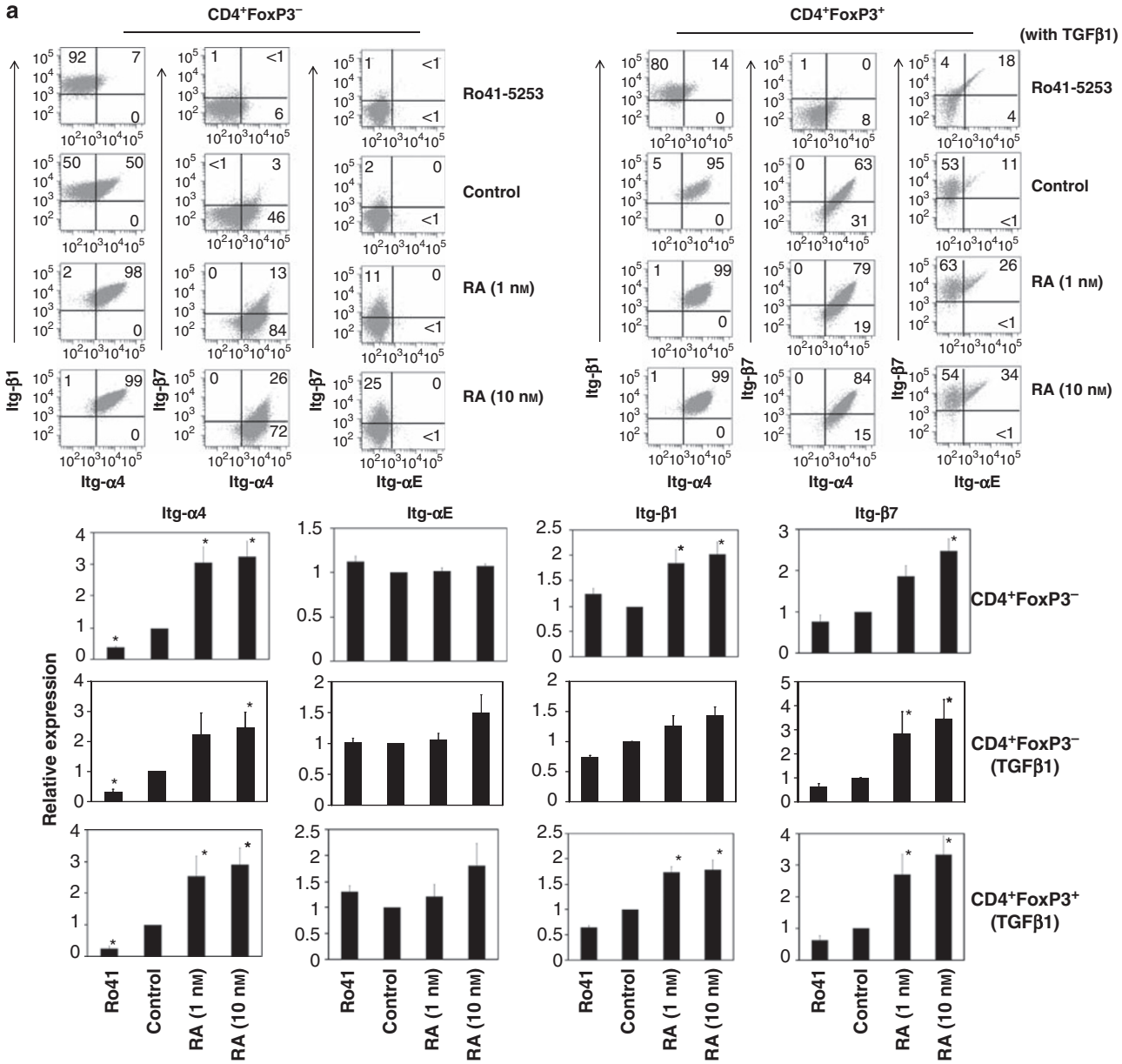
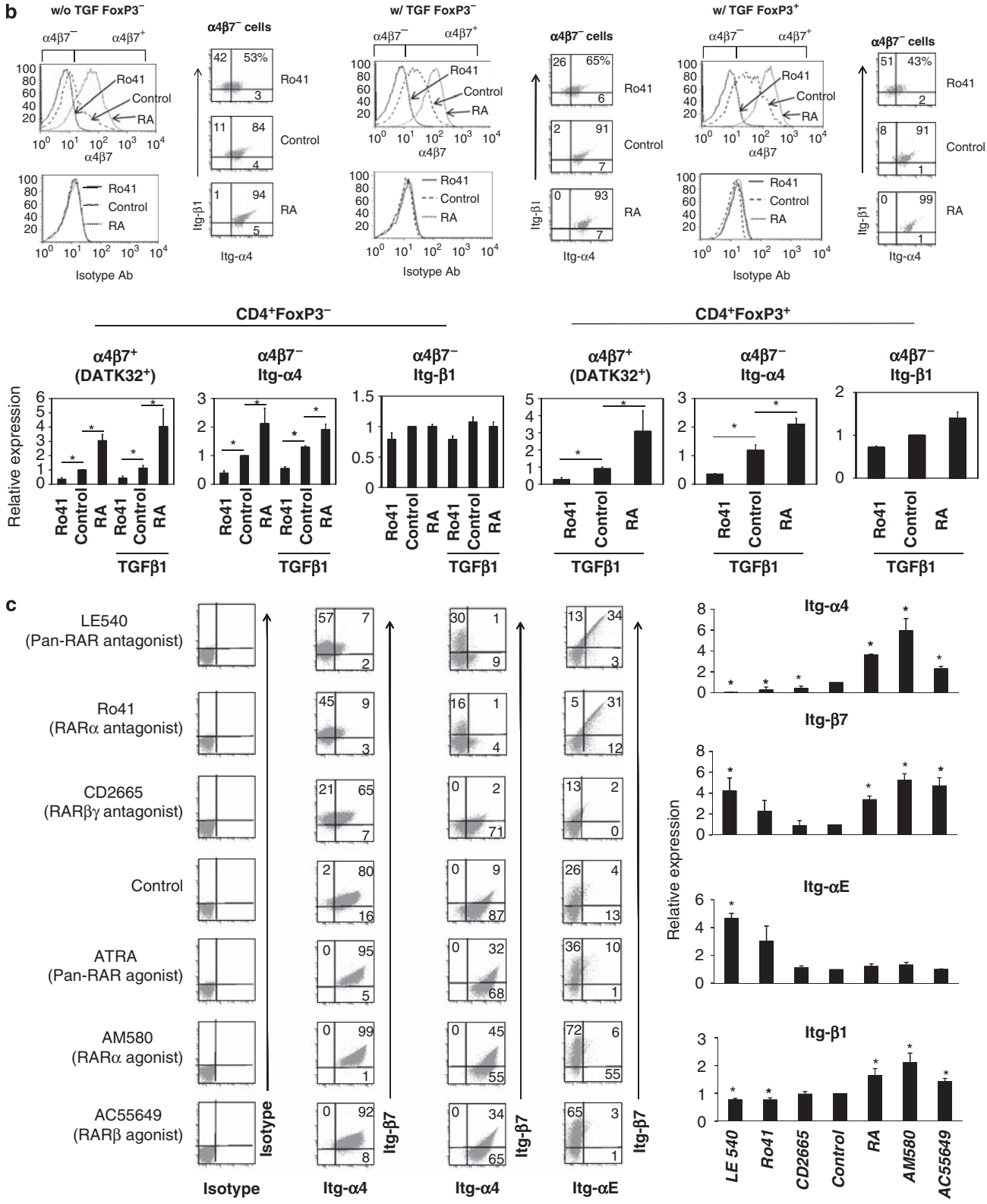


Figure 2 Expression of integrin proteins on the T-cell surface in response to a retinoic acid (RA) gradient. (**a**) Mouse CD4$^+$ T cells were activated in the absence or presence of Ro41 or RA for 6 to 7 days and cell surface expression of selected integrins was determined by flow cytometry. Transforming growth factor-β1 (TGFβ1; 1 ng ml^{-1}) was added to indicated cultures to determine any synergistic effects on induced FoxP3$^+$ and FoxP3$^-$ T cells. (**b**) Expression of Itg-α_4 and Itg-β_1 by $\alpha_4\beta_7{}^+$ and $\alpha_4\beta_7{}^-$ CD4$^+$ T cells treated with RA and/or TGFβ1. (**c**) Effects of various retinoic acid receptor-α (RAR) agonists and antagonists on expression of Itg-α_4, Itg-α_E, Itg-β_7, and Itg-β_1 by T cells. Expression levels were calculated based on mean fluorescence intensity, which indicates levels of surface antigen expression. Graphs show combined relative expression levels after normalization with the controls (n=3–6). *Significant differences from the controls.



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medium (**Figure 5b**). The background expression level of Itg-α_4 and Itg-β_7 was higher in the serum-containing medium compared with the serum-free medium. We also observed that Itg-α_4 mRNA expression was increased with the increasing RA signal (**Supplementary Figure S7** online). Itg-α_E transcription was increased with blocking with Ro41. Itg-β_7 mRNA

was highly expressed in CD8$^+$ T cells in all conditions but was further increased by RA. This response of CD8$^+$ T cells to RA is considered a minor difference from the CD4$^+$ T cells.

The effect of RA-dependent Itg-α_4 expression on T-cell migration

$\alpha_4\beta_1$ binds VCAM-1 and fibronectin, and $\alpha_4\beta_7$ binds MadCAM-1 and VCAM-1. Therefore, deficiency in Itg-α_4 expression due to RARα blockade would have an important functional consequence on the migration of T cells. We examined whether the T cells with decreased expression of Itg-α_4 due to RARα blockade would migrate normally on VCAM-1 *in vitro* (**Figure 6a**). The specificity of this migration was confirmed by PS/2 (an Itg-α4 blocking antibody)-dependent blocking of cell migration. Ro41-treated T cells were significantly defective in migration

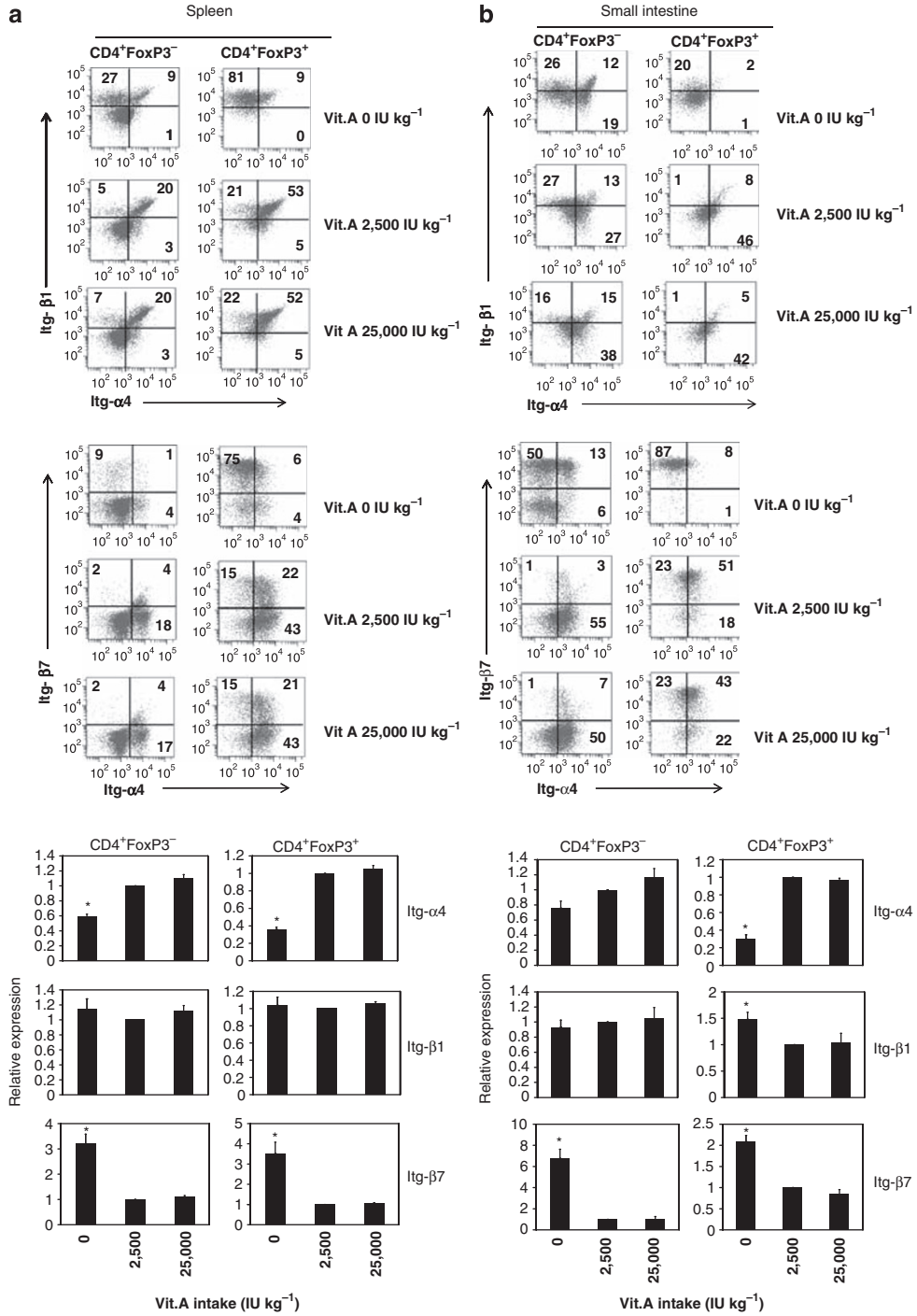

Figure 3 Expression of integrins in vitamin A deficiency. Expression of Itg-α_4 and Itg-β_7 by (**a**) spleen and (**b**) small intestinal lamina propria T-cell subsets in vitamin A-deficient, normal, and high mice. Expression of Itg-α_E and Itg-β_7 by (**c**) spleen and (**d**) small intestinal lamina propria T-cell subsets. Vitamin A-deficient, normal, and high mice were prepared, respectively, by feeding with special diets containing 0, 2,500, and 25,000 IU kg^{-1} for 12 to 13 weeks after birth. Representative and combined data (n=4) are shown. *Significant differences from the controls (2,500 U kg^{-1}).

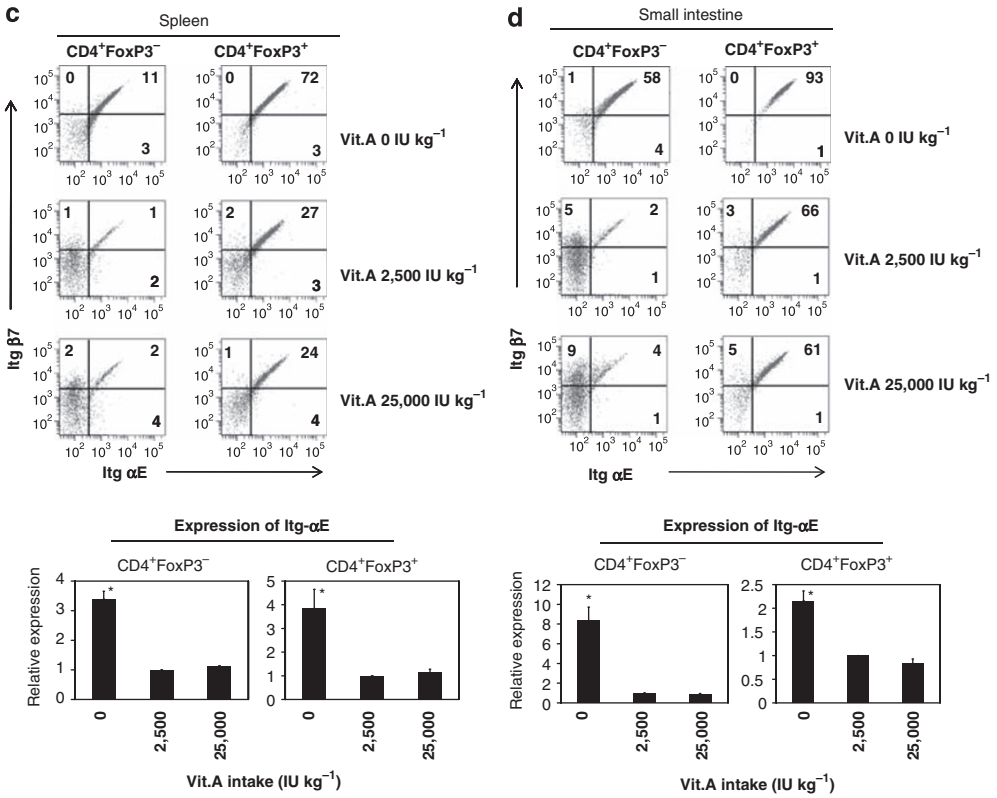

Figure 3 Continued.

through the VCAM-1-coated Transwell membrane. This occurred not only in spontaneous migration but also in stromal cell-derived factor-1/CXCL12-induced chemotaxis (**Figure 6a**). To determine the effect only on α4β1, we used T cells isolated from Itg-β7 knockout mice as well. A similar reduction in migration through the VCAM-1-coated Transwell membrane was observed for Ro41-treated T cells (**Figure 6b**).

We next assessed the *in vivo* migratory capacity of the Ro41-treated T cells compared with RA-treated T cells. Because CCR9, a major trafficking receptor to the small intestine,[33–36] is another receptor greatly induced by RA,[22] we used T cells from CCR9-deficient mice to rule out the effect of CCR9 on *in vivo* migration of T cells (**Figure 6c, d**). We found that Ro41-treated CCR9-deficient T cells were defective in migration to the intestine and Peyer's patches compared with RA-treated CCR9-deficient T cells (**Figure 6c**). Their migration to the spleen, mesenteric lymph node, peripheral lymph node, and lung was not affected. Our intravital study revealed that there is a pronounced difference between Ro41-treated T cells and control RA-treated T cells in adhesion to the endothelium of Peyer's patches (**Figure 6e**). Thus, the RA-induced expression of Itg-α_4 is functionally important for T-cell migration *in vivo*.

DISCUSSION

Expression of integrins is regulated at several levels of biological processes, including transcription and other post-transcriptional regulatory events, translation in endoplasmic reticulum, dimerization, and transportation from endoplasmic reticulum to the cell surface.[37] Transcription in response to specific induction signals has a central role in the expression of certain integrins. Another important factor for the expression of integrins is availability of heterodimerization partners because monomers cannot be expressed on the cell surface.[38,39] Availability of dimerization partners is particularly important for the coordinated expression of $\alpha_4\beta_1$ and $\alpha_4\beta_7$, which share the common α4 subunit. Similarly, expression of $\alpha_4\beta_7$ is linked to $\alpha_E\beta_7$ because of the common β_7 subunit.

We investigated the signals required for the induction of Itg-α_4 and related integrins. We found that Itg-α_4 is the integrin that is highly upregulated by RA (**Figure 7a**). Even at low residual concentrations in a regular medium containing 10% fetal bovine serum (~0.5 nM), RA is sufficient to induce Itg-α_4 transcription. This suggests that induction of Itg-α_4 chain can occur widely in the body at the plasma concentration of RA. This is different from CCR9, which is induced at higher levels (>5 nM) of exogenous RA. This difference in sensitivity to RA would limit the expression of CCR9 to the small-intestinal T cells, whereas α_4 integrins, particularly $\alpha_4\beta_1$, is more widely expressed on antigen-primed T cells in most tissues. Because Itg-α_4 is the common subunit for $\alpha_4\beta_1$ and $\alpha_4\beta_7$, RA provides a regulatory signal critical for the expression of the two integrin complexes. On the other hand, RA–vitamin A deficiency induces the expression of $\alpha_E\beta_7$ despite the fact that this molecule shares the Itg-β_7 chain with $\alpha_4\beta_7$. This confirms that RA is not required for transcription of Itg-α_E and Itg-β_7. Indeed, we found that Itg-α_E is mainly upregulated by a different signal provided by TGFβ1. Itg-β_7 is constitutively expressed and can be further induced by TGFβ1. We did not examine the roles of TGFβ isoforms other than TGFβ1 in integrin regulation. In support of our findings, it was previously reported that transcription of Itg-α_E and Itg-β_7 in a CD8$^+$ leukemic T cell line (TK-1) can be increased

by TGFβ1.[31] Whether TGFβ1 induces the expression of Itg-α_E and Itg-β_7 in primary naive CD4$^+$ T cells during antigen priming has not been determined despite the fact that natural and TGFβ1-induced FoxP3$^+$ T cells highly express $\alpha_E\beta_7$.[17,41]

Although both Itg-β_7 and Itg-β_1 are upregulated on the surface of T cells in response to RA, RA seems to have no essential role in transcription of these molecules. The increased expression of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ in response to RA is largely because of increased transcription and expression of Itg-α_4. Although we observed a certain increase of Itg-β_7 transcription in CD8 T cells in response to RA, this induction seems to be not important for $\alpha_4\beta_7$ expression, as Itg-β_7 is not a limiting factor. Thus, increased availability of Itg-α_4 leads to increased assembly of integrin complexes formed between pre-existing Itg-β_1 or Itg-β_7 chains and the RA-induced Itg-α_4 chain. This is supported by a recent publication by Shimizu group[40] that levels of Itg-β_1 expression can negatively

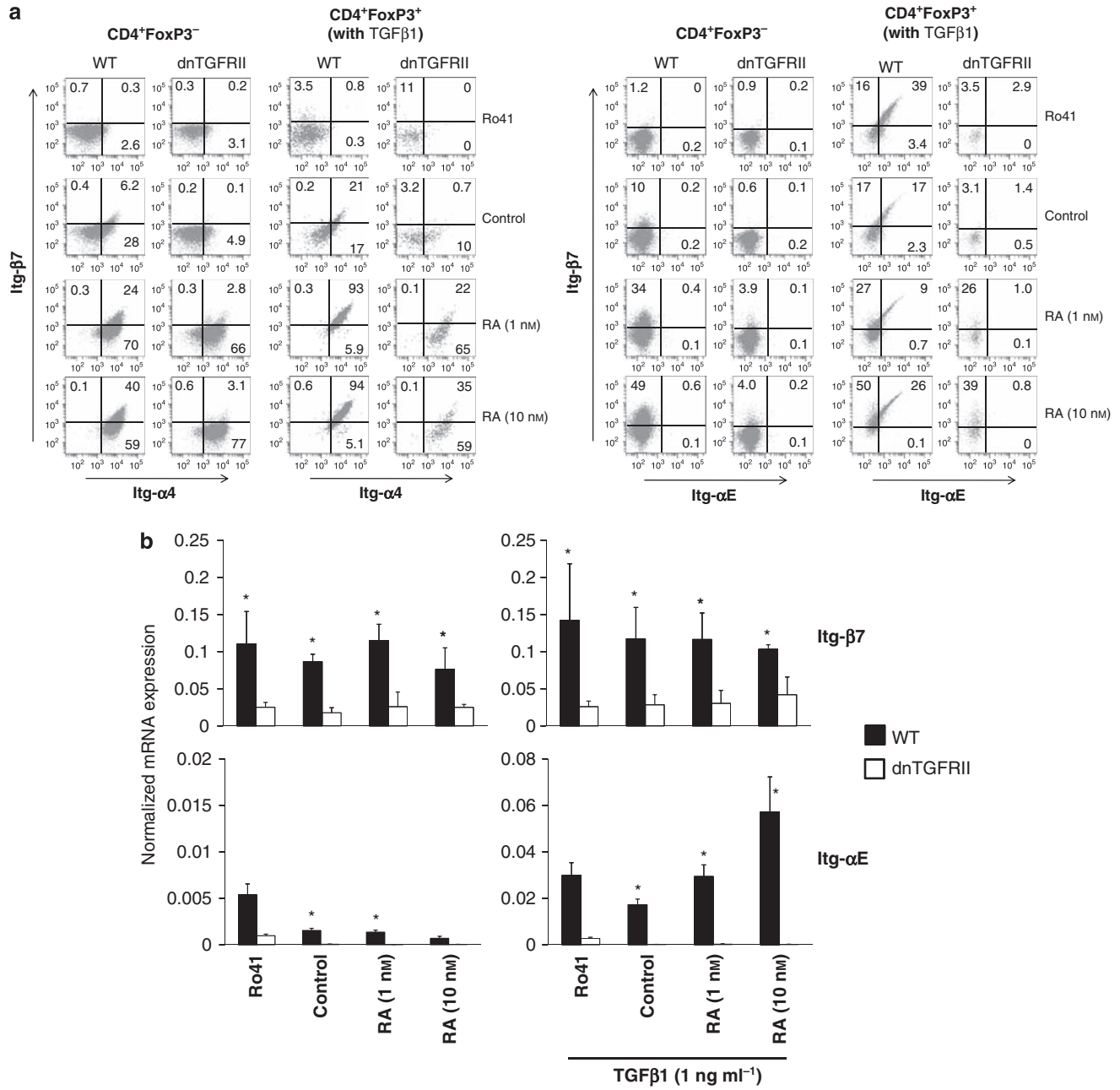

Figure 4 Transforming growth factor-β1 (TGFβ1) signal is required for expression of Itg-β_7 and Itg-α_E. (**a**) Surface expression of $\alpha_4\beta_7$ and $\alpha_E\beta_7$ in the presence and absence of TGFβ signal. A retinoic acid (RA) gradient is made with Ro41, RA (1 nM), and RA (10 nM) in 10% fetal bovine serum (FBS)-containing medium. TGFβ1 (1 ng ml^{-1}) was added to indicated cultures. Naive CD4$^+$ T cells, isolated from wild-type or dnTGFβRII mice, were cultured for 6 to 7 days in the different RA–TGFβ conditions. Representative data (n=4) are shown. (**b**) Expression of indicated Itg genes at the mRNA level is shown. Combined real-time PCR data with s.e.m. of three independent experiments is shown. (**c**) Expression of Itg-α_E and Itg-β_7 by the T cells in dnTGFβRII mice. (**d**) Expression of Itg-α_4 and Itg-β_1 by the T cells in dnTGFβRII mice. The graphs show combined data (percentage of positive cells among each T-cell subset) obtained from three different mice per group.

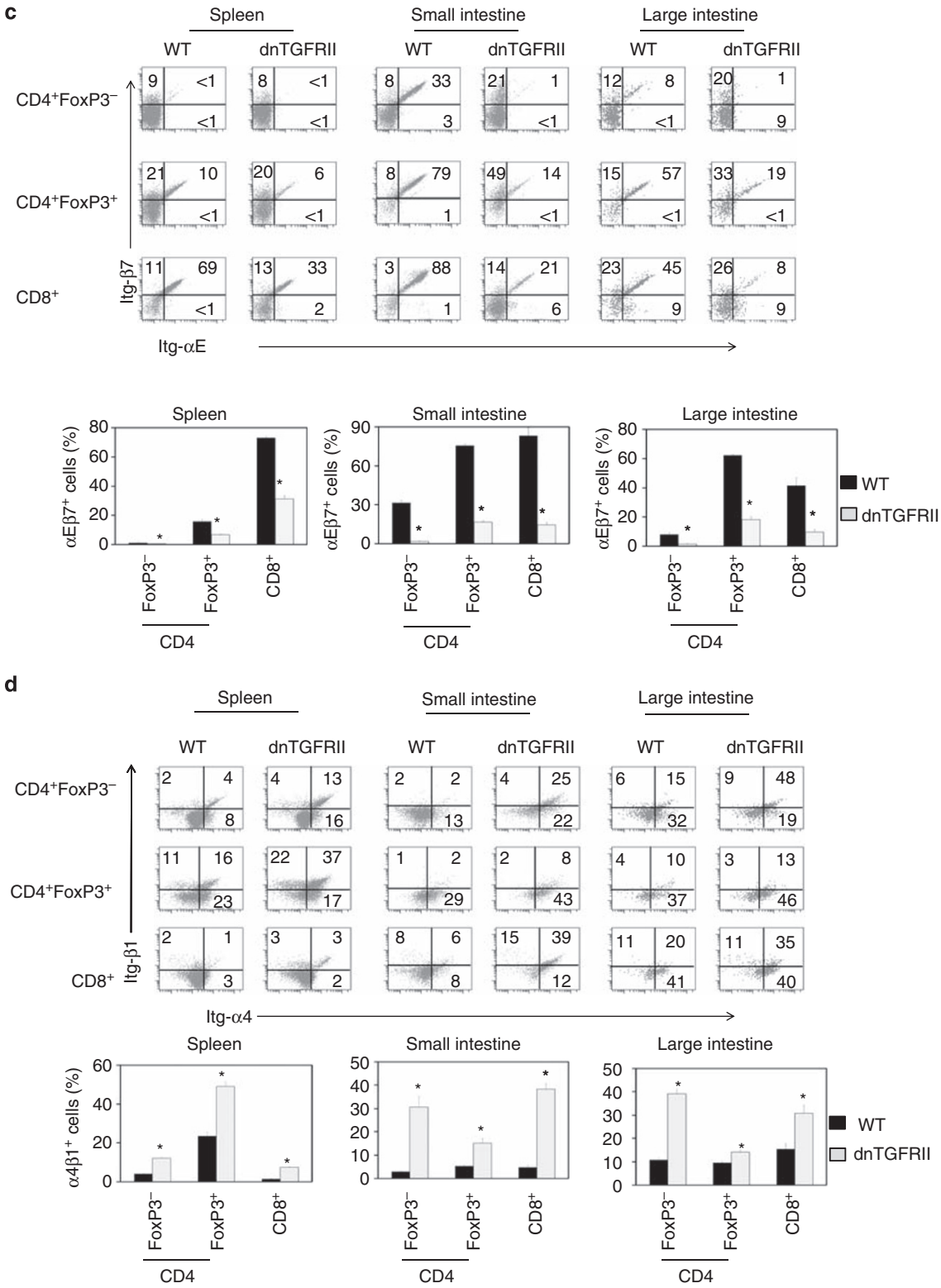

Figure 4 Continued.

affect $\alpha_4\beta_7$ expression through competition for Itg-α_4 molecules available for dimerization. Reciprocally, we found that decreased expression of Itg-β_7, as observed in T cells of CD4-dnTβRII mice, can lead to increased expression of $\alpha_4\beta_1$. In addition, we need to consider that there are many additional β_1 integrins besides $\alpha_4\beta_1$, which could further affect the regulation of the integrins. Thus, competition between Itg-β subunits is an important factor in the surface expression of α_4 integrins (**Figure 7b**).

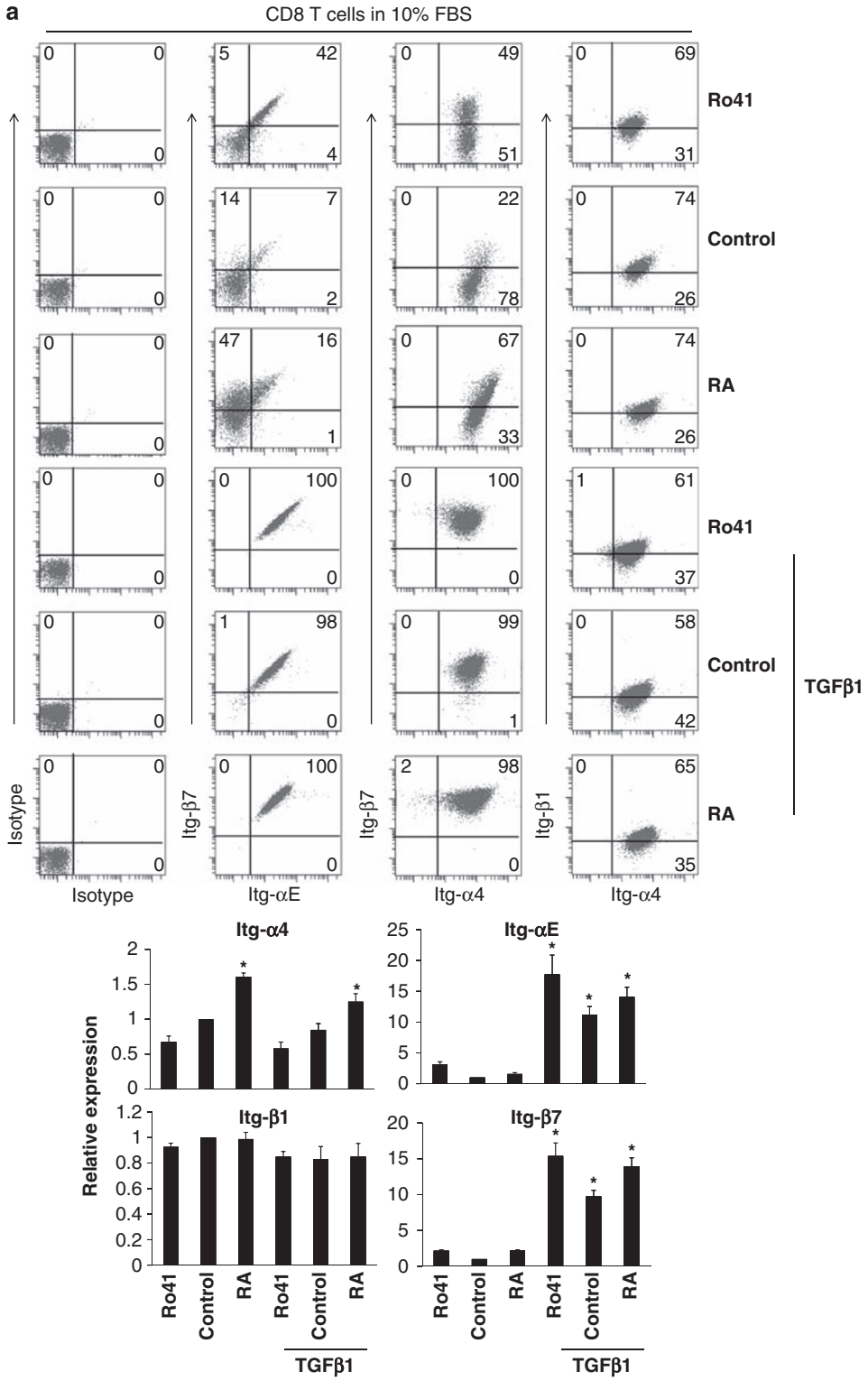

Figure 5 Regulation of integrins on CD8$^+$ T cells by retinoic acid (RA) and transforming growth factor-β1 (TGFβ1). Mouse CD8$^+$ T cells were activated in the absence or presence of Ro41 or RA for 5 to 6 days in (**a**) a 10% fetal bovine serum (FBS)-containing medium or (**b**) a serum-free medium, and cell surface expression of selected integrins was determined by flow cytometry. The small subset of CD8$^+$ FoxP3$^+$ T cells were excluded from the analysis. TGFβ1 (1 ng ml^{-1}) was added to indicated cultures to determine any synergistic effects. Graphs show combined relative expression levels after normalization for controls (n=3). *Significant differences from the controls.

The surface expression of the Itg-β_7 chain was greatly increased in response to RAR blockade or in vitamin A deficiency, because its pairing partner, the Itg-α_E subunit, is greatly induced in this condition in a manner dependent on the TGFβ1 signal. This upregulation of $\alpha_E\beta_7$ in RA deficiency is probably because of increased availability of Itg-β_7 molecules for pairing with Itg-α_E when Itg-α_4 expression

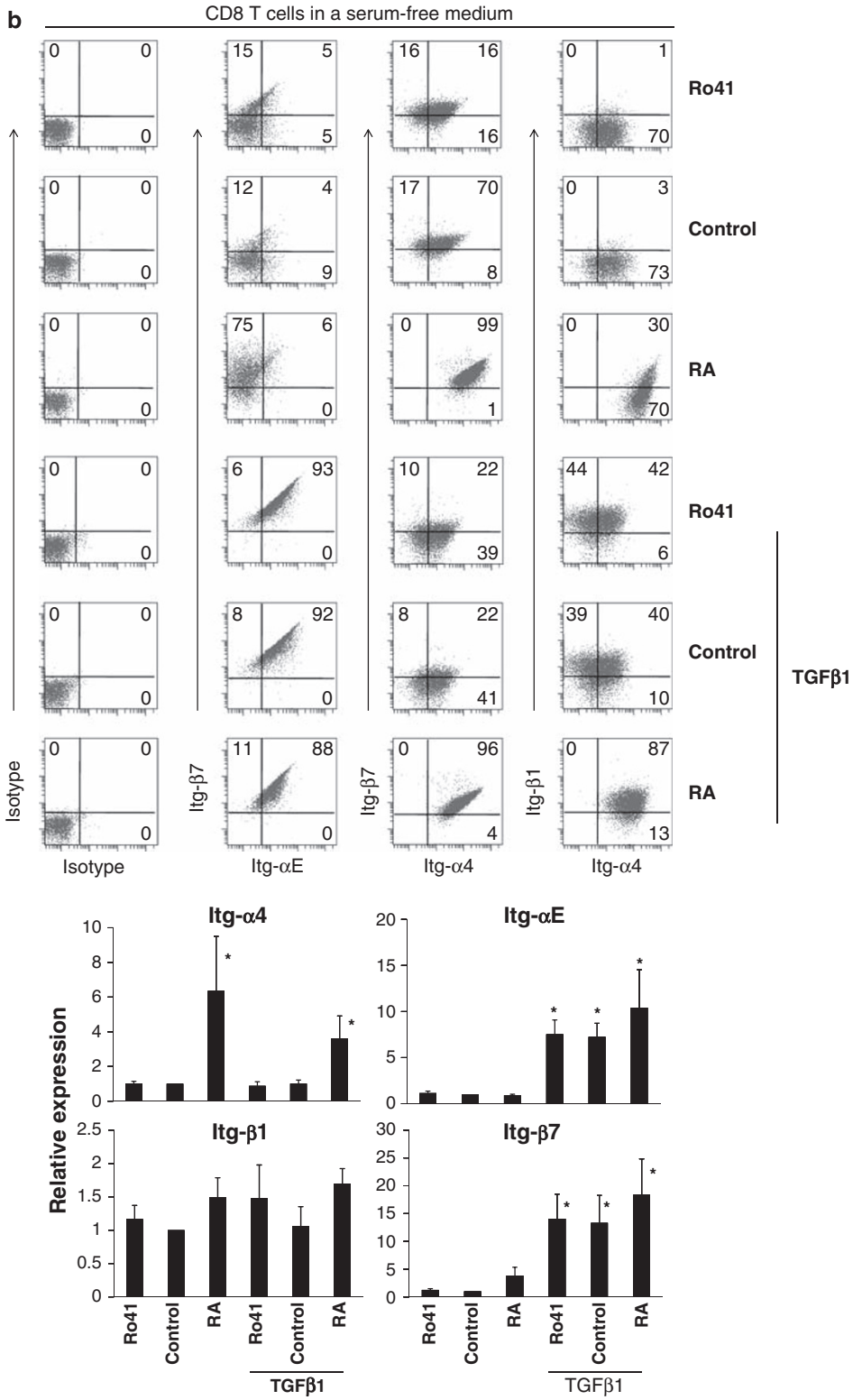

Figure 5 Continued.

is severely decreased (**Figure 7b**). Another mechanism is active induction of Itg-α_E transcription in RA deficiency.

The two integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$ have critical roles in lymphocyte migration for both homeostatic and inflammatory purposes. Normal expression of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ is required for mounting effective immunity and inducing chronic inflammation. Moreover, Itg-α_4 is an effective target for treatment of inflammatory diseases.[42,43] A side effect of blocking Itg-α_4 is increased susceptibility to infection.[44,45] Our results show that $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins are greatly decreased on

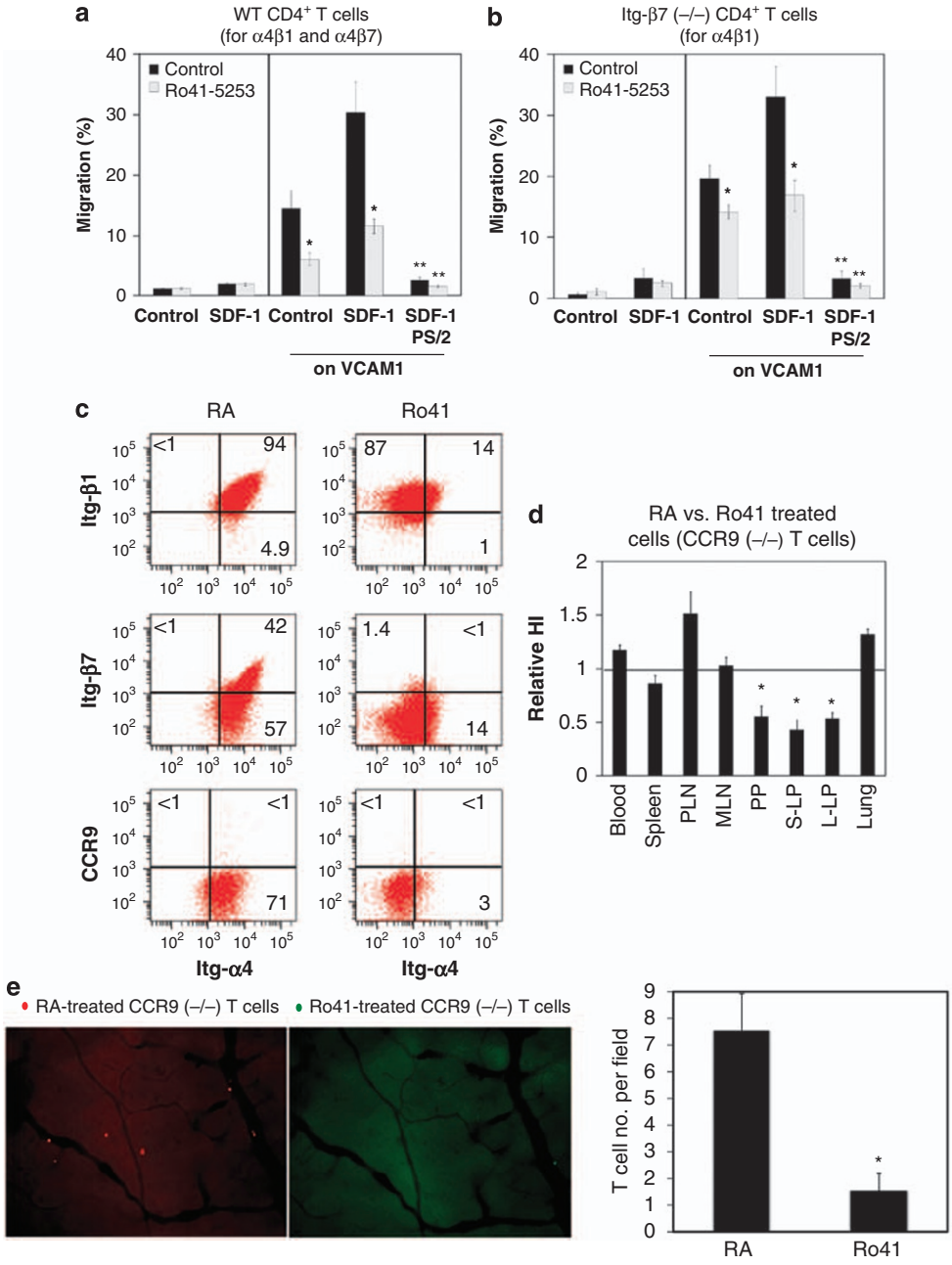

Figure 6 Functional effects of Itg-α_4, induced by low concentrations of retinoic acid (RA), on migration of T cells *in vitro* and *in vivo*. (**a, b**) Migration of Itg-α_4-low and normal T cells through vascular cell adhesion molecule 1 (VCAM1)-coated Transwells in response to stromal cell-derived factor-1 (SDF-1) or control medium was examined. Wild-type T cells can express both $\alpha_4\beta_1$ and $\alpha_4\beta_7$, whereas Itgβ_7 (−/−) T cells express $\alpha_4\beta_1$ but not $\alpha_4\beta_7$. Combined data of three independent experiments are shown. Naive CD4$^+$ T cells, isolated from (**a**) wild-type or (**b**) Itgβ7 (−/−) mice were cultured in the presence of control medium or Ro41 to prepare control and Itgα_4-low T cells, respectively. PS/2, an Itg-α_4 blocking monoclonal antibody, was used to block the Itg-α_4-dependent migration. *Significant differences from control T cells. **Significant differences from the SDF-1 groups. (**c**) Surface phenotype of CD4$^+$ T cells used for the *in vivo* homing study. Chemokine (C-C motif) receptor 9 (CCR9; −/−) naive CD4$^+$ T cells were cultured in the presence of RA or Ro41 to prepare control and Itg-α_4-low T cells. CCR9 (−/−) naive CD4$^+$ T cells were used to exclude the effect of CCR9 (another trafficking receptor induced by RA) on migration. (**d**) Migration of Itg-α_4-low T cells *in vivo*. A 20-h short-term *in vivo* homing assay to various organs including the peripheral lymph node (PLN), mesenteric lymph node (MLN), Peyer's patches (PP), small intestinal lamina propria (S-LP), and large intestinal lamina propria (L-LP) was performed. Homing indices <1 indicate decreased homing compared with control T cells. Combined data (n=4–6). (**e**) Intravital microscopy was performed to visualize the migrating Itg-α_4-low and control CCR9 (−/−) T cells to Peyer's patches immediately after the T-cell transfer. A representative set of data of three independent experiments is shown. Error bars indicate s.d. of the number of cells per field (number of cells rolling, arrested, and migrated) in Peyer's patches in ~20 image frames. *Significant decreases in migration.

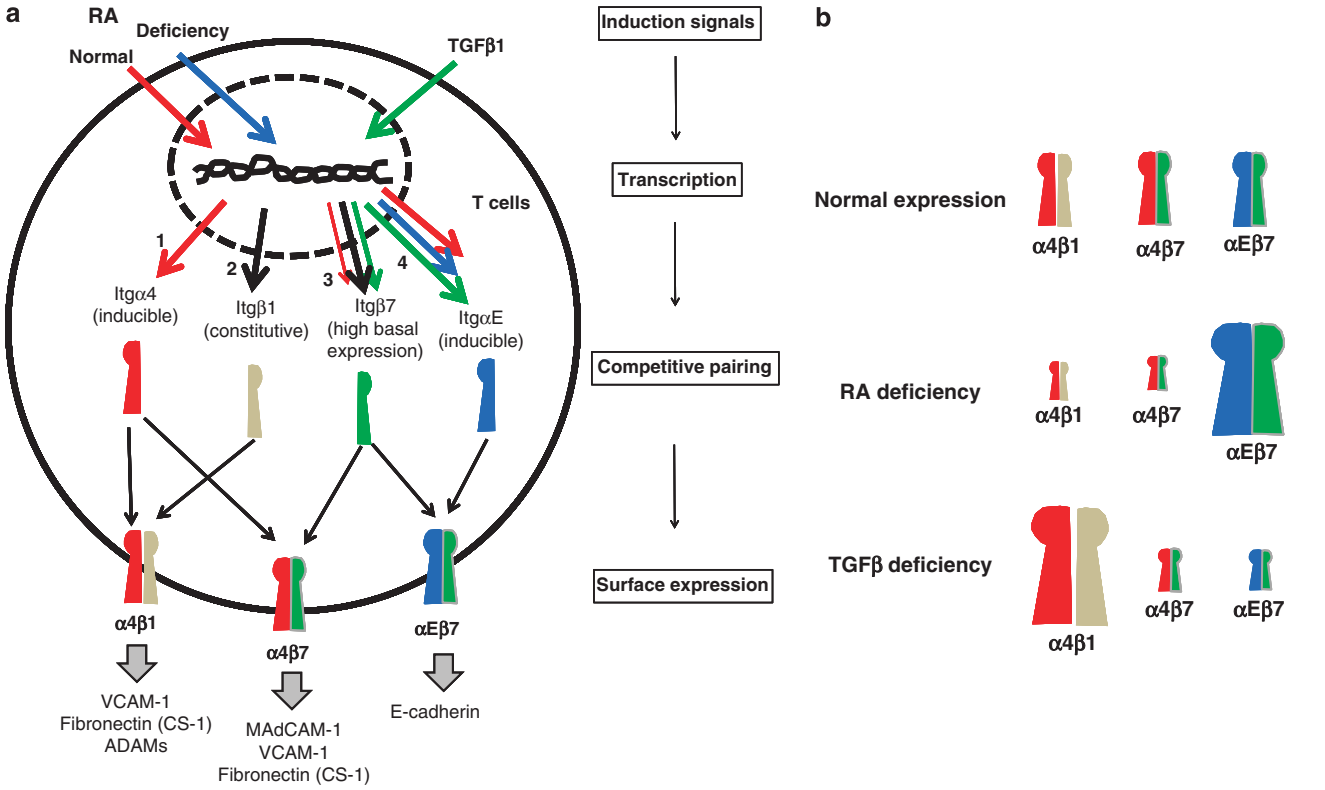

Figure 7 Coordinated regulation of the expression of $\alpha_4\beta_1$, $\alpha_4\beta_7$, and $\alpha_E\beta_7$ by retinoic acid (RA) and transforming growth factor-β1 (TGFβ1) in T cells. (**a**) RA positively induces Itg-α_4 (red lines) but RA paucity induces Itg-α_E (blue lines). In contrast, Itg-α_E is induced by TGFβ1 (green lines), whereas Itg-β_1 is constitutively expressed by activated T cells. Itg-β_7 is constitutively expressed but can be further induced by TGFβ1. In addition, RA seems to increase Itg-β_7 expression in $CD8^+$ T cells. Because of the heterodimerization requirement, expression of the three integrins is influenced by RA and TGFβ signals in combination. $\alpha_4\beta_1$ can be induced by RA alone, whereas high expression of $\alpha_4\beta_7$ requires both RA and TGFβ1. High expression of $\alpha_E\beta_7$ requires TGFβ1. In the intestine and other tissues, in which RA is available at optimal concentrations, high expression of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ by T cells occurs. (**b**) In vitamin A deficiency, decreases in expression of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ but increases in $\alpha_E\beta_7$ occur because of decreased Itg-α_4 and increased Itg-α_E expression. In a TGFβ signal deficiency, $\alpha_4\beta_1$ is overexpressed because the expression of $\alpha_4\beta_7$ (and $\alpha_E\beta_7$) is suppressed, freeing up Itg-α_4 molecules for pairing with Itg-β_1. Reciprocally, $\alpha_4\beta_7$ is overexpressed when $\alpha_4\beta_1$ is not expressed because of Itg-β_1 deficiency.[40] This regulatory mechanism operates in most T cells, including $FoxP3^+$, $CD4^+$, and $CD8^+$ T cells. The size of integrins in the diagram signifies the amount of expression.

the surface of T cells in vitamin A deficiency. Decreased expression of these integrins leads to defective migration to various mucosal tissues such as the lung and intestine. It is likely that the increased susceptibility to infection in vitamin A-deficient individuals is, in part, because of the decreased expression of the α_4 integrins and consequentially lowered effector functions of immune cells.[46]

The changes in expression levels of $\alpha_4\beta_1$, $\alpha_4\beta_7$, and $\alpha_E\beta_7$ in different retinoid–vitamin A status occur in all of the T-cell subsets examined in this study such as $CD4^+$ T cells and $CD8^+$ T cells. It is particularly notable that expression of the integrins by RA and TGFβ1 is more clearly regulated in $FoxP3^+$ T cells. We believe that this is, in part, because of the fact that TGFβ1 is required for induction of both FoxP3 and integrins (i.e., Itg-α_E and Itg-β_7). Therefore, the RA- and TGFβ-dependent regulation of the integrins would have profound effects on migration and function of $FoxP3^+$ T cells as well. Defective migration of $FoxP3^+$ T cells can promote inflammatory diseases and explains, in part, the increased inflammation in vitamin A deficiency.[47]

METHODS

Cell isolation and culture. $CD4^+$ T cells were isolated from pooled single-cell suspensions of spleen, mesenteric lymph nodes, and peripheral lymph node with the $CD4^+$ T-cell isolation kit (Miltenyi Biotec, Auburn, CA). Cells expressing CD8, CD19, CD25, CD44, and CD69 were further depleted to obtain naive $CD4^+$ T cells (purity of ~95%). Total $CD8^+$ T cells were isolated using the $CD8^+$ T-cell isolation kit (Miltenyi Biotec), and were further depleted for CD4, CD19, CD25, CD44, and CD69 cells to obtain naive ($CD8^+CD25^-CD44^-CD69^-$) $CD8^+$ T cells (purity of ~93%). Naive cells were activated for 5 to 6 days with complete RPMI-1640 medium containing concanavalin A (2.5 μg ml^{-1}) and human interleukin-2 (100 U ml^{-1}) in the presence or absence of one of the agonists or antagonists: RA (= At-RA; 1 or 10 nM from Sigma Aldrich, St Louis, MO), Ro41-5253 (hereafter called Ro41, 500 nM, purchased from Biomol, Farmingdale, NY), LE540 (500 nM, pan-RAR antagonist; Wako Chemical, Richmond, VA), CD2665 (500 nM, selective RAR-β/γ antagonist; Tocris Bioscience, Ellisville, MO), AM580 (20 nM, RARα agonist; Tocris), and AC55649 (100 nM, RARβ2 agonist; Tocris). Optimal concentrations of these reagents were determined by a preliminary titration study. Human TGFβ1 (1 ng ml^{-1}) was used when indicated. For the experiment in **Supplementary Figure S4** online, cycloheximide (10 μg ml^{-1}; Enzo, Farmingdale, NY) was used. The T cells were also cultured in a serum-free

medium (HL-1 from Lonza, Allendale, NJ) for 5 to 6 days with the T-cell activation–Expansion kit (anti-CD3 and CD28 beads: 6 µl per million cells; Miltenyi Biotec) and human interleukin-2 (100 U/ml^{-1}) in the presence of RA (10 nM), Ro41 (100 nM), and/or TGFβ1 (1 ng ml^{-1}).

Animals and generation of vitamin A-deficient or sufficient mice. All the experiments with animals in this study were approved by the Purdue animal care and use committee. CCR9-deficient mice were described previously.[48] Itg-β_7 (−/−) mice (C57BL/6-Itgb7^{tm1Cgn}/J) and dominant form of TGFRII transgenic mice (B6.Cg-Tg(Cd4-TGFBR2)16Flv/J) were purchased from the Jackson Laboratory (Bar Harbor, ME). For generation of mice with excessive, normal, or deficient vitamin A status, BALB/c mice (Jackson Laboratory) were kept on custom diets based on AIN-93G containing high (25,000 IU kg^{-1}; tenfold higher than the normal dietary range), normal (2,500 IU kg^{-1}), or low (0 IU kg^{-1}) (Harlon Teklad, Indianapolis, IN: TD-06528, 00158, and 07267) levels of vitamin A as previously described.[49] The pups were weaned at 4 weeks of age and maintained on the same diets for additional 9 weeks. Vitamin A deficiency was verified by defective CCR9 expression by small intestinal T cells as described previously.[49]

Flow cytometry. Itg-α_4 was stained sequentially with purified anti-mCD49d antibody (clone 9C10; BioLegend) followed by biotin anti-rat IgG2a (clone MRG2a-83; BioLegend) and Streptavidin-PerCP/Cy5.5 (BioLegend, San Diego, CA). To detect expression of Itg-β_1, β_7, and α_E, respectively, antibodies to mCD29 (clone HMß1-1), mItg-β_7 (clone FIB504), and mCD103 (clone 2E7) were used. Anti-mLPAM-1 (DATK32) was used to detect $\alpha_4\beta_7$. When indicated, cells were stained for intracellular mFoxP3 with an antibody (clone FJK-16s; eBioscience, San Diego, CA). Stained cells were analyzed using a BD Canto II (BD Bioscience, San Diego, CA).

Homing experiment. Wild-type or CCR9-deficient T cells were prepared by culturing with RA or Ro41 and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) or tetramethylrhodamine isothiocyanate (TRITC). The two cell types (5 million cells for each cell type per mouse) were co-injected through a tail vein into C57BL/6 mice. After 20 h, the mice were killed and single-cell suspensions were prepared from selected organs after collagenase digestion as previously described.[50] The numbers of the injected CFSE$^+$ or TRITC$^+$ cells present in each organ was determined with flow cytometry. The relative homing index was determined according to the formula: homing index for organ A = (no. of TRITC$^+$ cells in organ A)/(no. of CFSE$^+$ cells in organ A) ÷ (no. of TRITC$^+$ cells in input)/(no. of CFSE$^+$ cells in input).

VCAM-1-dependent chemotaxis. Chemotaxis was performed with Transwells (3.0 µm pores; Corning, Corning, NY). The Transwells were coated with mouse VCAM-1 (100 µg ml^{-1}; R&D Systems, Minneapolis, MN) in 50 µl of $NaHCO_3$ (0.1 M, pH 8.0) by incubating overnight at 4 °C, and blocked with 2% bovine serum albumin for 30 min at room temperature. RA- (CFSE-labeled) and Ro41 (TRITC-labeled)-treated T cells (0.5×10^5 cells each) in 100 µl of chemotaxis buffer (RPMI-1640, 0.5% bovine serum albumin) were loaded onto the upper chamber. Stromal cell-derived factor-1α (100 ng ml^{-1}, R&D Systems) was added to the lower chamber. Neutralizing anti-mItg-α4 monoclonal antibody (PS/2, 5 µg ml^{-1}) was added to block the cells in the upper chamber when indicated. The cells were allowed to migrate for 4 h at 37 °C, and the cells that migrated to the lower chamber were counted with flow cytometry.

Intravital microscopy to monitor T-cell migration into gut lymphoid tissues. CCR9-deficient T cells, cultured with RA or Ro41 and labeled with CFSE or TRITC, were injected through a tail vein into C57BL/6 mice. Immediately after the injection of the labeled cell into anesthetized mice, Peyer's patches were exposed and observed in a custom-designed intravital device equipped with a Leica (Bannockburn, IL) DMI 3000B fluorescent microscope and a dynamic ultra low light fluorescence camera (Retiga-EXi; QImaging, Surrey, BC, Canada). The images were acquired every 0.5 s in sequence with the QCapture Pro6.0 software (Surrey, BC, Canada). Images were analyzed frame by frame for the presence of cells adhering to the endothelium, which include the cells under both rolling and sticking. Data from at least 30 images were averaged to obtain numbers of cells adhering to the Peyer's patch endothelium.

Microarray and data analysis. RNA, isolated from cultured CD4$^+$ T cells, was hybridized to Mouse 430 2.0 chips (Affymetrix, Santa Clara, CA) by the Purdue Genomics Laboratory staff. These arrays contain over 39,000 complementary DNA spots corresponding to mouse sequence verified transcripts. Raw intensity values were obtained (GeneChip Operating Software, Affymetrix) and normalized with the expression values of a housekeeping gene (β-*actin*). Selection and filtering of high-quality genes was based on a twofold or greater differential in expression up or down between two conditions of comparison. Further selection was based on reproducibility between duplicated experiments, and transcripts without consistent results were dismissed. The gene expression values were visualized with the multiplot module of the GenePattern genomic analysis platform (www.broad.mit.edu/cancer/software/genepattern). The raw and processed array data have been deposited at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20500.

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation assay was performed using a kit following the manufacturer's instruction (Upstate Biotechnology, Lake Placid, NY). In brief, 2×10^6 naive CD4$^+$ T cells were cultured in the presence or absence of RA (10 nM) or Ro41 (500 nM). Concanavalin A (2.5 µg ml^{-1}) and human interleukin-2 (25 U ml^{-1}) were used to activate T cells. The cells were cultured for 4 to 5 days, fixed in paraformaldehyde, and made into cell lysates. The chromosomal DNA–protein complex was sonicated to generate DNA fragments with their size ranging from 200 to 1000 bp. DNA–protein complexes were immunoprecipitated using 4 µg of polyclonal antibody against mouse RARα (Santa Cruz Biotech, Santa Cruz, CA). Genomic DNA enriched with antibodies against RARα was uncross-linked and analyzed by PCR for detection of RAREs in the mouse *Itg*-α_4 gene promoter with the following primer pairs: 5′-TACTTTGATGTCTATTTCTCTGG-3′ and 5′-GGATAGCAAGAAGTGCTGTCC-3′ (RARE1); 5′-AAGCCATCAGTGCTTCTCACC-3′ and 5′-GGAGAGACC TTGTGTCAAAGAA-3′ (RARE2); 5′-ATTCAGCTTGGCTGACAGGGA-3′ and 5′-TCC TTTTGCCTCTGCCTGCC-3′ (RARE3); 5′-TCCTATAA GCTTTGTTTTCAGCC-3′ and 5′-ACAACGTTTTATCTCATAA GTAATC-3′ (RARE4/5); 5′-AAAACTACCCATCTACTATAAAC AA-3′ and 5′-CAACTCAAACTCCTATTAAGTTCT-3′ (RARE6); 5′-TCTGAACCTAGCAACTGCCAC-3′ and 5′-CCACTCCCAG TCTTTTGGAGA-3′ (RARE7). Real-time PCR detection was performed with a 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR green Master Mix (Applied Biosystems).

Statistical analyses. Student's paired and unpaired *t*-tests were used to compare the significance of the differences between two groups of related or unrelated data. The *P*-values of $\leqslant 0.05$ were considered significant.

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

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DISCLOSURE

The authors declared no conflict of interest.

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