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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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Abstract

α4 and β7 integrins such as α4β1, α4β7 and αEβ7 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-α4 subunit by both $CD4^+$ and $CD8^+$ T cells requires the retinoic acid signal. In contrast, transcription of *Itg-αE* genes is induced by the TGFβ1 signal. Expression of *Itg-β7* is constitutive but can be further increased by TGFβ1. Consistently, expression of α4-containing integrins is severely suppressed in vitamin A deficiency with a compensatory increase of αEβ7, whereas expression of Itg-αE and Itg-β7 is decreased in TGFβ-signal deficiency with a compensatory increase in α4β1. The retinoic acid-mediated regulation of α4 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 α4, a subunit of α4β1 (CD49d–CD29) and α4β7, is a major target of intervention in treating inflammatory diseases through blocking leukocyte migration.1, 2 α4β1, also known as very late antigen 4 (VLA4), is expressed by T cells, B cells, monocytes and eosinophils.3, 4 α4β7, once called lamina propria–associated molecule 1, is expressed by T cells and B cells in mucosal tissues.5, 6 α4β1 and α4β7 each bind its major counter receptor vascular-cell adhesion molecule 1 (VCAM-1) and mucosal adhesion-cell adhesion molecule 1 (MAdCAM-1) respectively. α4β7 also binds VCAM-1 and fibronectin, but at reduced affinity.7 The α4 integrins are involved in both rolling and firm adhesion of leukocytes on endothelial cells.8, 9 α4β1 is involved in leukocyte migration to diverse tissues including mucosal tissues, bone marrow, splenic follicles and inflamed tissues, while α4β7 has a more specific role in lymphocyte migration to the gut and associated lymphoid tissues.10–14 αEβ7, an integrin related to α4β1 and α4β7, binds E-cadherin expressed on epithelial cells.

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15 αEβ7 is involved in localization and function of effector as well as 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. RA is required for differentiation of promyelocytes into neutrophils.19–21 RA promotes the generation of small intestine-homing T and B cells.22, 23 In this regard, a severe paucity of T cells and IgA-producing B cells occurs in the intestine 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 Retinoic acid specifically induces the expression of a small intestine-homing chemokine receptor CCR9 and a mucosal tissue-homing integrin molecule α4β7.22

It has been unclear how the mucosal integrins such as α4β1, α4β7, and αEβ7 are coordinately expressed. Through genome-wide gene expression and functional studies, we identified that expression of the Itg-α4 chain in T cells requires RA, while optimal transcription of *Itg-αE* and to a lesser degree *Itg-β7* genes requires the TGFβ signal. We provide detailed evidence that functional expression of Itg-α4- or Itg-αE-subunit-containing integrins by T cells is coordinately regulated by RA and TGFβ1. The impact of the integrin regulation pathways on T cell migration in physiological settings is discussed.

Results

RA selectively induces the transcription of *Itg-α4* gene

In order to understand the function of RA in regulation of mucosal integrin expression in T cells, we activated naïve $CD4^+$ 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 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-α4 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-β1, Itg-β7 and Itg-αE 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-α4* gene is responsive to RA in transcription, while the expression of *Itg-β1* and *Itg-β7* was not significantly affected by the presence or absence of the RA signal (Figure 1C). Itg-α4 transcription was further increased by exogenous RA at 1 and 10 nM. Interestingly, the real-time PCR assay revealed that Itg-αE mRNA was up-regulated when Ro41 was used, which the microarray study failed to reveal.

We performed a chromatin immunoprecipitation (ChIP) assay to assess potential binding of nuclear RARα to the regulatory region of the Itg-α4 gene. Based on a DNA sequence analysis, we found a total of 7 putative retinoic acid response elements (RAREs). The ChIP

assay revealed that one of the putative RAREs is a real binding site for RARα (Figure 1D). RA enhanced the binding while Ro41 decreased the binding.

In addition to *Itg-α4*, many genes are up or down-regulated in T cells in response to RA. These genes are listed in Table 1 and Figure S1 in the supplementary material section. 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 P450, family 26, subfamily b, polypeptide 1 (CYP26B1), dehydrogenase/ reductase member 3 (DHRS3), and leucine zipper transcription factor-like 1 (LZTFL1) are induced by RA, while serine (or cysteine) proteinase inhibitor clade B member 1a (SERPINB1) is suppressed by RA (Figure S1). GRTP1 is a GTPase activating protein that increases GTPase activity of Rab3A.26 CYP26B1 is a cytochrome P 450 that catabolizes retinoic acid.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-α4.

T cell activation in the presence of RA induces surface expression of both α4β1 and α4β7

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 if the α4 subunit induced by RA is required for all α4 subunit-containing integrins. We examined if RA would have significant effects on surface expression of α4β1 and α4β7 (Figure 2A). T cells activated in the presence of Ro41 lost expression of Itg-α4. In contrast, Itg-β1 was expressed at high levels even with Ro41. Surface expression of Itg-β7 was enhanced with RA. Induction of surface Itg-α4 expression by RA is independent of exogenous TGFβ1 in culture. Itg-αE 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-α4* gene transcription is a driving force in the surface expression of both Itg-β7 and Itg-β1.

We assessed also the expression of the α4β7 heterodimer complex with the DATK32 antibody (Figure 2B). By gating out the $\alpha4\beta7^{+}$ cells, expression of the α4β1 complex by $\alpha4\beta7^{-}$ T cells was also determined. It was apparent that expression of both α4β7 and α4β1 was increased in response to the RA signal.

To gain more insights into the RAR receptor usage, we utilized 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). While both AM580 and AC55649 induced the expression of Itg-α4, AM580 was more potent even at a lower dose (20 nM). Ro41 was more potent than CD2665. LE540 was most potent in induction of Itg-β7 and Itg-αE. Overall, this information suggests that both the RARα and RARβ/γ receptors can regulate expression of the integrins, but RARα appears to have a larger role.

Flow cytometric determination of the perfect co-expression of Itg-αE and Itg-β7 or Itg-α4 and Itg-β7 revealed sharp needle-like double positive populations that may be viewed as the result of autofluorescence or miscompensation in flow cytometry. Employing appropriate isotype controls, we confirmed that these double positive cells indeed have perfect co-expression of integrin subunits (Figure S2).

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 (Figure S3). We confirmed that RA induces Itg-α4. The T cells of the control group expressed Itg-α4 at levels similar to those of the Ro41-treated group. This rules out the possibility that the decreased Itg-α4 expression by Ro41 in a regular medium is due to an unexpected agonistic effect of Ro41.

We further examined the stability of the expressed integrins induced by RA utilizing cycloheximide, a protein biosynthesis inhibitor (Figure S4). We found that the induced integrins (α4β7, Itg-α4, Itg-β7, Itg-β1, and Itg-αE) were stable on the cell surface for at least 12 hours following the treatment of cycloheximide.

T cell expression of Itg-α4 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-α4 was greatly decreased on both $FoxP3^-$ and $FoxP3^+$ $CD4^+$ T cells in vitamin A deficient compared to control mice (Figure 3A). This decrease was relatively more severe on $FoxP3^+$ T cells compared to $FoxP3^-$ T cells. Itg-β1expression was not affected by different vitamin A status. Itg-β7 was, unexpectedly, induced in vitamin A deficiency. In the small intestine, Itg-α4 was again decreased in vitamin A deficiency with almost no or minor change in expression of Itg-β1 (Figure 3B). Itg-β7 was again strongly induced in vitamin A deficiency in the small intestine.

Increased surface expression of Itg-β7 in vitamin A deficiency is a mystery given the current perception that RA would induce Itg-β7 expression. We hypothesize that the enhanced expression of Itg-β7 in vitamin A deficiency would be the result of increased expression of it's the other dimerization partner Itg-αE in vitamin A deficiency. When we examined the expression of Itg-αE and Itg-β7, it was clear that most Itg-β7 molecules were co-expressed on T cells with the Itg-αE molecules in vitamin A deficiency (Figure 3C and D). This co-expression was evident on T cells in both spleen and small intestine. The tight co-expression of Itg-αE and Itg-β7 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 α4β1 and α4β7 in the mice with normal vitamin A status to those expressing αEβ7 in vitamin A deficiency (Figure S5).

The TGFβ signal is required for functional expression of Itg-β7 and Itg-αE

A question critical for expression of both α4β7 and αEβ7 is what would regulate the transcription of the *Itg-β7* gene. TGFβ is implicated in up-regulation of Itg-β7 in a cytotoxic

lymphoma cell line.31 We examined if the TGFβ signal is required for expression of Itg-β7 in primary $CD4^+$ T cells utilizing 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. Naïve T cells from the dnTGFβRII mice were ineffective in surface expression of Itg-β7 in response to RA, while expression of Itg-α4 was induced normally (Figure 4A). The induction of Itg-β7 on wild type $FoxP3^+$ T cells was even higher while 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-αE and Itg-β7 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 (Figure S6). 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-αE and Itg-β7 in the wild type and dnTGFβRII T cells cultured with RA or Ro41. We observed that dnTGFβRII T cells fail to express Itg-αE and Itg-β7 transcripts (Figure 4B). These results demonstrate that the TGFβ1 signal is required for expression of Itg-β7 and Itg-αE 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-αE and Itg-β7 was decreased on $CD4^+$ T cells in the spleen, small intestine, and large intestine (Figure 4C). Instead, there were increases in T cells expressing α4β1, which appears to be a compensatory response to the Itg-β7 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 and D on $CD8^+$ T cells show that optimal expression of αEβ7 requires the TGFβ signal. We determined further if expression of Itg-α4 and other Itg chains is induced by RA in $CD8^+$ T cells. Similar to $CD4^+$ T cells, Itg-α4 was strongly induced in response to RA (Figure 5A). Expression of Itg-αE and Itg-β7 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 medium (Figure 5B). The background expression level of Itg-α4 and Itg-β7 was higher in the serum-containing medium compared to the serum free medium. We observed also that Itg-α4 mRNA expression was increased with the increasing RA signal (Figure S7). Itg-αE transcription was increased with blocking with Ro41. Itg-β7 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 impact of RA-dependent Itg-α4 expression on T cell migration

α4β1 binds VCAM-1 and fibronectin, and α4β7 binds MAdCAM-1 and VCAM-1. Therefore, deficiency in Itg-α4 expression due to RARα blockade would have an important functional consequence on migration of T cells. We examined if the T cells with decreased

expression of Itg-α4 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 through the VCAM-1-coated Transwell membrane. This occurred not only in spontaneous migration but also in SDF-1/CXCL12-induced chemotaxis (Figure 6A). To determine the impact only on α4β1, we utilized T cells isolated from Itg-β7 KO 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 to 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 impact of CCR9 on *in vivo* migration of T cells (Figure 6C and D). We found that Ro41-treated CCR9-deficient T cells were defective in migration to the intestine and Peyer's patches compared to RA-treated CCR9-deficient T cells (Figure 6C). Their migration to the spleen, mesenteric lymph node (MLN), peripheral lymph node (PLN) 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-α4 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 plays a central role in expression of certain integrins. Another important factor for expression of integrins is availability of hetero-dimerization partners because monomers cannot be expressed on the cell surface.38, 39 Availability of dimerization partners is particularly important for coordinated expression of α4β1 and α4β7, which share the common α4 subunit. Similarly, expression of α4β7 is linked to αEβ7 because of the common β7 subunit.

We investigated the signals required for induction of Itg-α4 and related integrins. We found that Itg-α4 is the integrin that is highly up-regulated by RA (Figure 7A). RA even at low residual concentrations in a regular medium containing 10% fetal bovine serum (~0.5 nM) is sufficient to induce Itg-α4 transcription. This suggests that induction of Itg-α4 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, while α4 integrins, particularly α4β1, is more widely expressed on antigen-primed T cells in most tissues. Because Itg-α4 is the common subunit for α4β1 and α4β7, RA provides a regulatory signal critical for expression of the two integrin complexes. On the other hand, RA/vitamin A deficiency induces the expression of αEβ7 despite the fact that this molecule shares the Itg-β7 chain with α4β7. This confirms that RA is not required for transcription of Itg-αE and Itg-β7. Indeed, we found that Itg-αE is mainly up-regulated by a different signal provided by

TGFβ1. Itg-β7 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-αE and Itg-β7 in a $CD8^+$ leukemic T cell line (TK-1) can be increased by TGFβ1.31 Whether TGFβ1 induces expression of Itg-αE and Itg-β7 in primary naïve $CD4^+$ T cells during antigen priming has not been determined despite the fact that natural and TGFβ1-induced $FoxP3^+$ T cells highly express αEβ7.17, 41

While both Itg-β7 and Itg-β1 are up-regulated on the surface of T cells in response to RA, RA appears to have no essential role in transcription of these molecules. The increased expression of α4β1 and α4β7 in response to RA is largely due to increased transcription and expression of Itg-α4. While we observed a certain increase of Itg-β7 transcription in CD8 T cells in response to RA, this induction appears to be not important for α4β7 expression as Itg-β7 is not a limiting factor. Thus, increased availability of Itg-α4 leads to increased assembly of integrin complexes formed between pre-existing Itg-β1 or Itg-β7 chains and the RA-induced Itg-α4 chain. This is supported by a recent publication by Shimizu group that levels of Itg-β1 expression can negatively affect α4β7 expression through competition for Itg-α4 molecules available for dimerization.40 Reciprocally, we found that decreased expression of Itg-β7, as seen in T cells of CD4-dnTβRII mice, can lead to increased expression of α4β1. In addition, we need to consider that there are many additional β1-integrins besides α4β1, which could further affect the regulation of the integrins. Thus, competition between Itg-β subunits is an important factor in surface expression of α4 integrins (Figure 7B).

The surface expression of the Itg-β7 chain was greatly increased in response to RAR blockade or in vitamin A deficiency because its pairing partner, the Itg-αE subunit, is greatly induced in this condition in a manner dependent on the TGFβ1 signal. This up-regulation of αEβ7 in retinoic acid deficiency is probably due to increased availability of Itg-β7 molecules for pairing with Itg-αE when Itg-α4 expression is severely decreased (Figure 7B). Another mechanism is active induction of Itg-αE transcription in retinoic acid deficiency.

The two integrins α4β1 and α4β7 play critical roles in lymphocyte migration for both homeostatic and inflammatory purposes. Normal expression of α4β1 and α4β7 is required for mounting effective immunity and inducing chronic inflammation. Moreover, Itg-α4 is an effective target for treatment of inflammatory diseases.42, 43 A side effect of blocking Itg-α4 is increased susceptibility to infection.44, 45 Our results demonstrate that α4β1 and α4β7 integrins are greatly decreased on 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, due to the decreased expression of the α4 integrins and consequentially lowered effector functions of immune cells.46

The changes in expression levels of α4β1, α4β7 and αEβ7 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, due to the fact that TGFβ1 is required for induction of both FoxP3 and integrins (i.e. Itg-αE and Itg-β7). Therefore, the RA and TGFβ-dependent regulation of the integrins would have profound impacts 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 (MLN) and peripheral lymph node (PLN) with the CD4$^+$ T cell isolation kit (Miltenyi Biotec, Auburn, CA). Cells expressing CD8, CD19, CD25, CD44 and CD69 were further depleted to obtain naïve CD4$^+$ T cells (purity of ~95%). Total CD8$^+$ T 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 naïve (CD8$^+$CD25$^-$CD44$^-$CD69$^-$) CD8$^+$ T cells (purity of~ 93%). Naïve cells were activated for 5 to 6 days with complete RPMI 1640 medium containing concanavalin A (2.5 μg/ml) and hIL-2 (100 U/ml) in the presence or absence of one of the agonists or antagonists: RA (=At-RA; 1 or 10 nM from Sigma Aldrich), Ro41–5253 (hereafter called Ro41, 500 nM, purchased from Biomol), LE540 (500 nM, pan-RAR antagonist, Wako Chemical), CD2665 (500 nM, selective RAR-β/γ antagonist, Tocris Bioscience), 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. hTGF-β1 (1 ng/ml) was used when indicated. For the experiment in Figure S4, cycloheximide (10μg/ml; Enzo) was used. The T cells were cultured also in a serum free medium (HL-1 from Lonza) for 5–6 days with the T cell activation/Expansion kit (anti-CD3 and CD28 beads: 6μl/million cells: Miltenyi Biotec) and hIL-2 (100 U/ml) in the presence of RA (10nM), Ro41 (100nM), and/or TGF-β1 (1 ng/ml).

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-β7 (−/−) 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. 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; 10-fold higher than the normal dietary range), normal (2,500 IU/kg) or low (0 IU/kg) (Harlan Teklad 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-α4 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). To detect expression of Itg-β1, β7 and αE respectively, antibodies to mCD29 (Clone HMββ1-1), mItg-β7 (clone FIB504), and mCD103 (clone 2E7) were used. Anti-mLPAM-1 (DATK-32) was used to detect α4β7. When indicated, cells were stained for intracellular mFoxP3 with an antibody (clone FJK-16s, eBioscience). Stained cells were analyzed using a BD Canto II (BD Bioscience).

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 each cell type/mouse) were co-injected via a tail vein into C57BL/6 mice. 20 hours later, the mice were sacrificed 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 (HI) for organ A = [(# of $TRITC^+$ cells in organ A) / (# of $CFSE^+$ cells in organ A) ÷ (# of $TRITC^+$ cells in input) / (# of $CFSE^+$ cells in input).

VCAM-1-dependent chemotaxis

Chemotaxis was performed with Transwells (Corning, 3.0 μm pores). The Transwells were coated with mouse VCAM-1 (R&D; 100 μg/ml) in 50 μl of $NaHCO_3$ (0.1 M, pH 8.0) by incubating overnight at 4 °C, and blocked with 2% of BSA 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 (RPMI1640, 0.5 % BSA) were loaded onto the upper chamber. SDF-1α (100 ng/ml, R&D Systems, Minneapolis, MN) was added to the lower chamber. Neutralizing anti-mItg-α4 monoclonal antibody (PS/2, 5 μg/ml) 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 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 via 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 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 sec in sequence with the QCapture Pro6.0 software. 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, Inc.) by the Purdue Genomics Laboratory staff. These arrays contain over 39,000 cDNA spots corresponding to mouse sequence verified transcripts. Raw intensity

values were obtained (GCOS, Affymetrix, Inc.) and normalized with the expression values of a housekeeping gene (β-actin). Selection and filtering of high quality genes was based on a two-fold 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 (ChIP)

A ChIP assay was performed using a kit following the manufacturer's instruction (Upstate Biotechnology, Inc., Lake Placid, NY). Briefly, 2×10^6 naïve $CD4^+$ T cells were cultured in the presence or absence of RA (10 nM), or Ro41 (500 nM). Concanavalin A (2.5 μg/ml) and hIL-2 (25 U/ml) were used to activate T cells. The cells were cultured for 4–5 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). Genomic DNA enriched with antibodies against RARα was uncross-linked and analyzed by PCR for detection of retinoic acid response elements (RAREs) in the mouse *Itg-α4* gene promoter with the following primer pairs: 5'-TAC.TTT.GAT.GTC.TAT.TTC.TCT.GG-3' and 5'-GGA.TAG.CAA.GAA.GTG.CTG.TCC-3' (RARE1); 5'-AAG.CCA.TCA.GTG.CTT.CTC.ACC-3' and 5'-GGA.GAG.ACC.TTG.TGT. CAA.AGA.A-3' (RARE2); 5'-ATT.CAG.CTT.GGC.TGA.CAG.GGA-3' and 5'-TCC. TTT.TGC.CTC.TGC.CTG.CC-3' (RARE3); 5'-TCC.TAT.AAG.CTT.TGT.TTT.CAG.CC-3' and 5'-ACA.ACG.TTT.TAT.CTC.ATA.AGT.AAT.C-3' (RARE4/5); 5'-AAA.ACT. ACC.CAT.CTA.CTA.TAA.ACA.A-3' and 5'-CAA.CTC.AAA.CTC.CTA.TTA.AGT.TCT-3' (RARE6); 5'-TCT.GAA.CCT.AGC.AAC.TGC.CAC-3' and 5'-CCA.CTC.CCA. GTC.TTT.TGG.AGA-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. *P* values ≤ 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

RA	all-trans retinoic acid
Itg	integrin
CFSE	carboxyfluorescein diacetate succinimidyl ester
TRITC	tetramethylrhodamine isothiocyanate
VCAM-1	vascular-cell adhesion molecule 1
Ro41	Ro41-5253.

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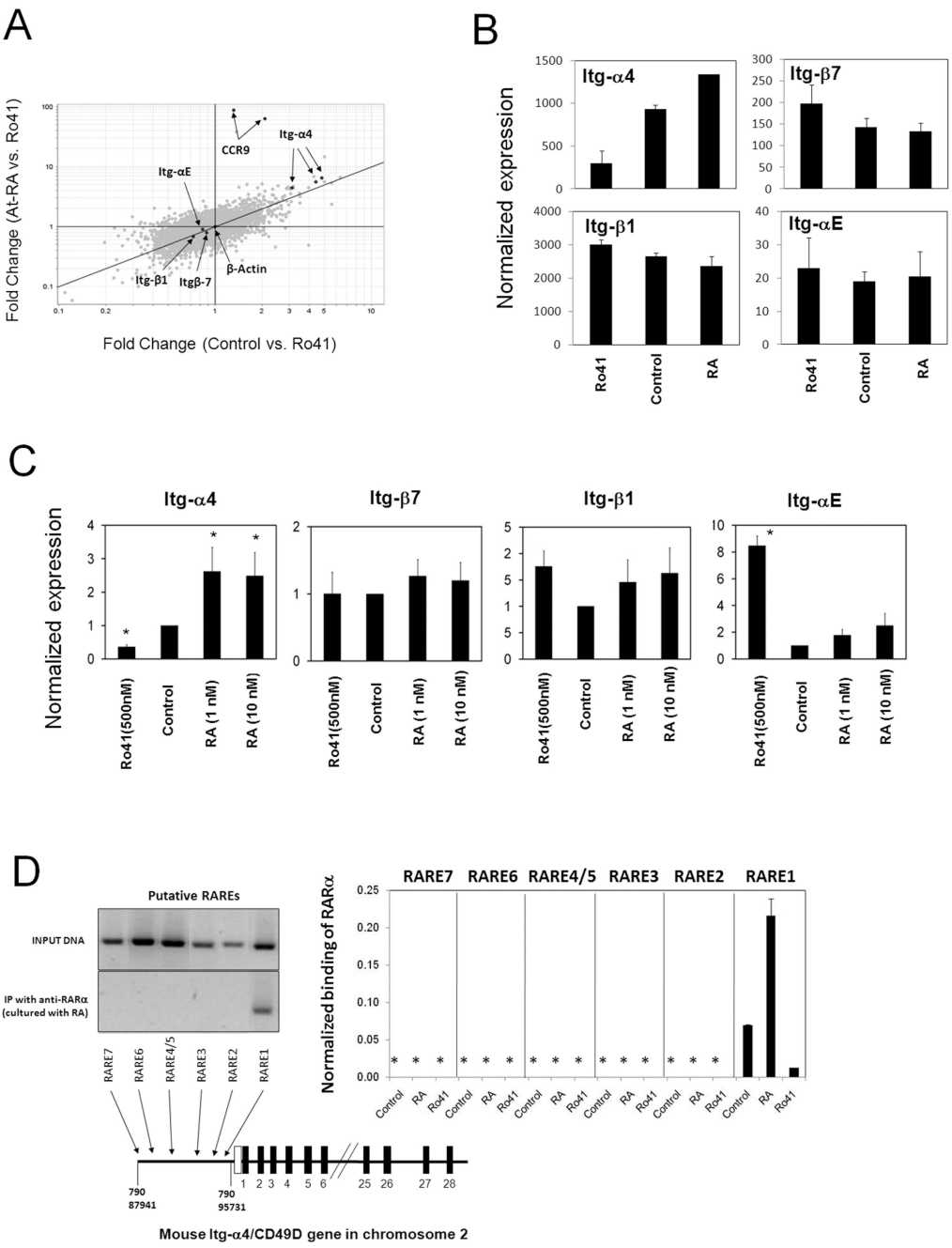
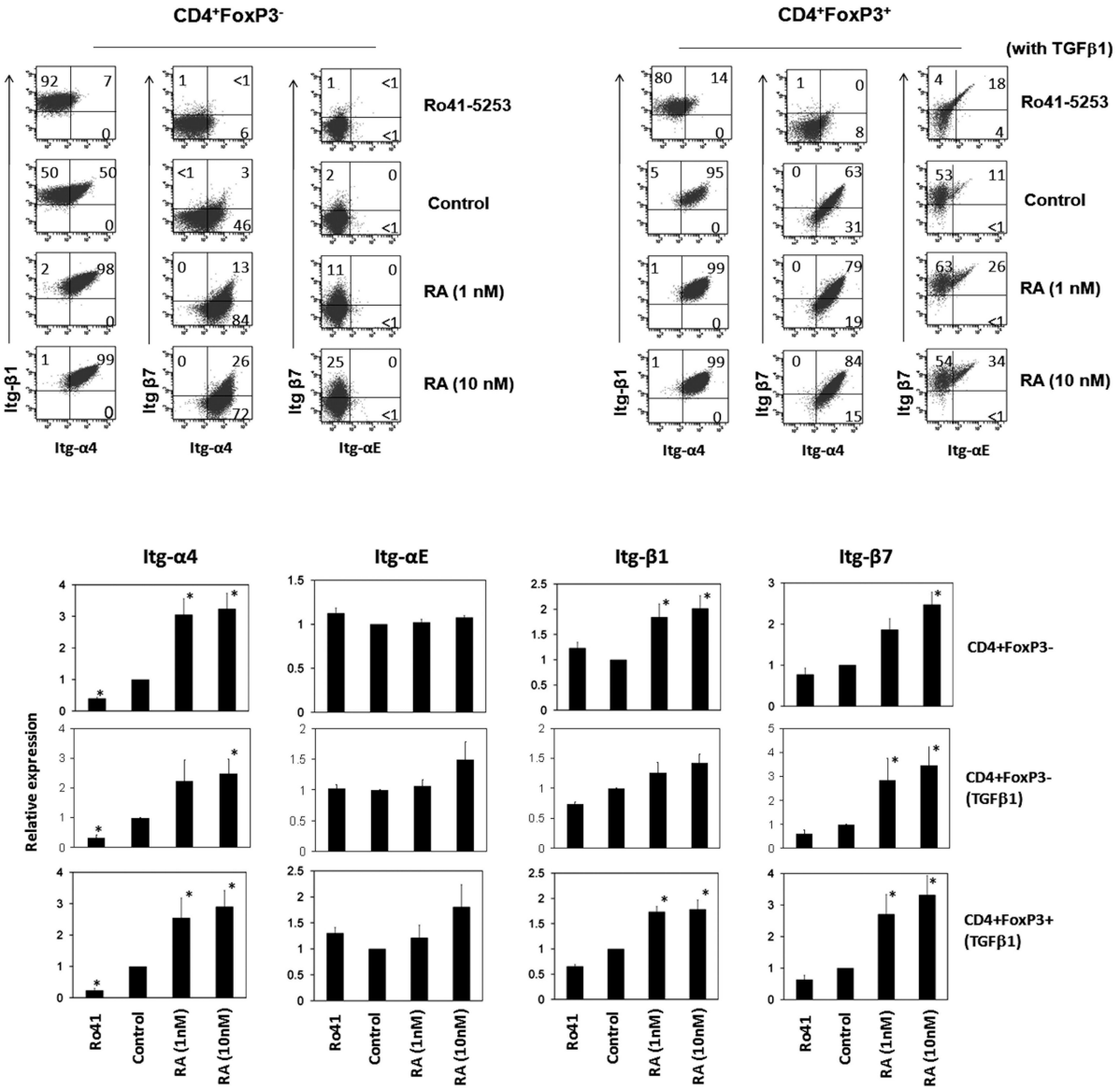


Figure 1. Retinoic acid- and RARα-dependent transcription of the *Itg-α4* gene, but not *Itg-β7*, *Itg-β1* and *Itg-αE* genes in CD4$^+$ T cells

(A) A dot plot showing genes up-regulated in response to high and low concentrations of RA. An Affymetrix microarray was used to determine the levels of gene expression. 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"). 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 4 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 responsive elements (RARE) in the 5' upstream regulatory region of the *Itg-α4* gene. A ChIP assay was performed to determine RARα binding to RARE candidates on the 5' upstream regulatory region of the *Itg-α4* gene. RA (10 nM) was used. A representative data set from 3 independent experiments is shown. *Undetectable (panel D).

A



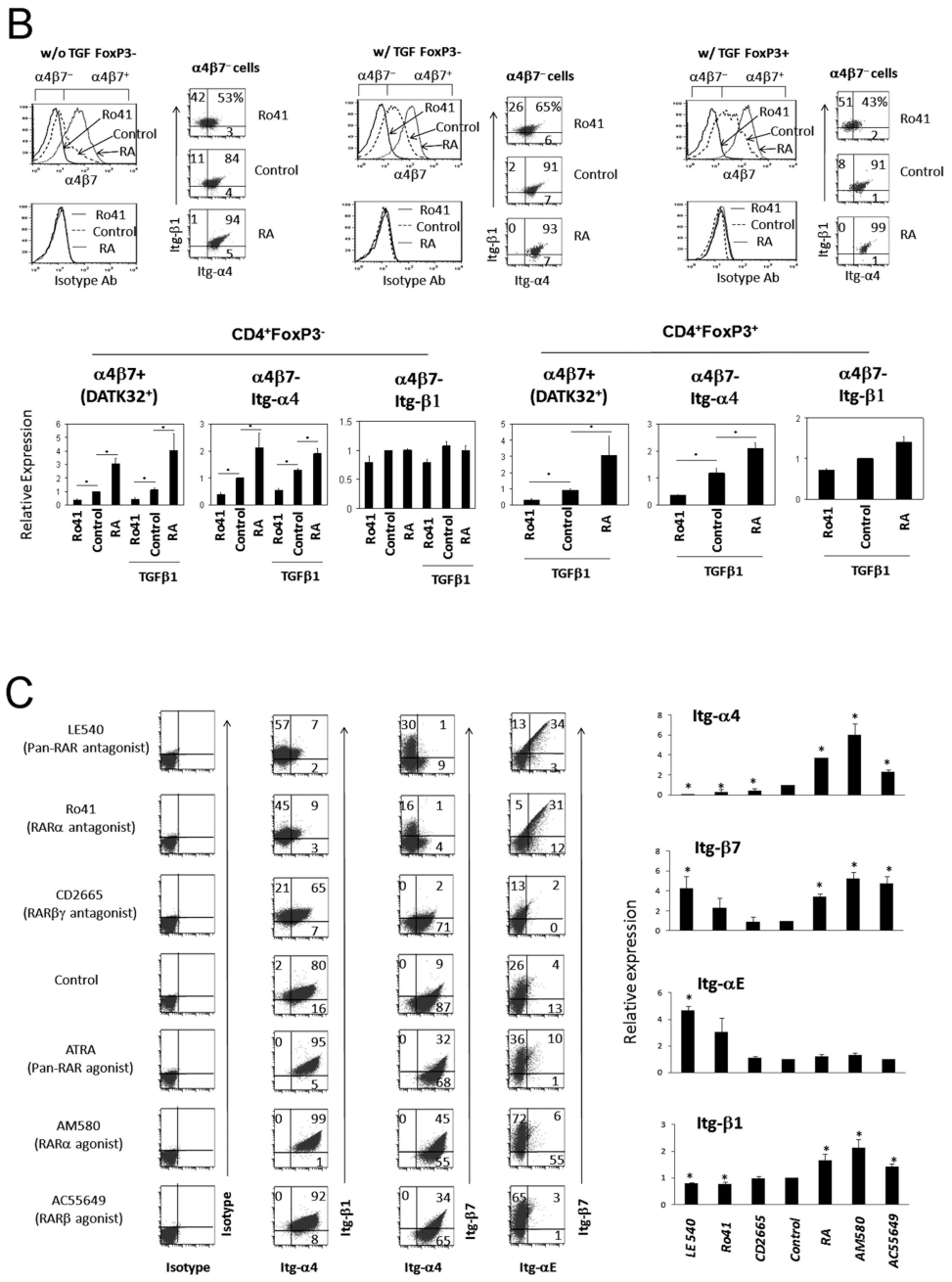
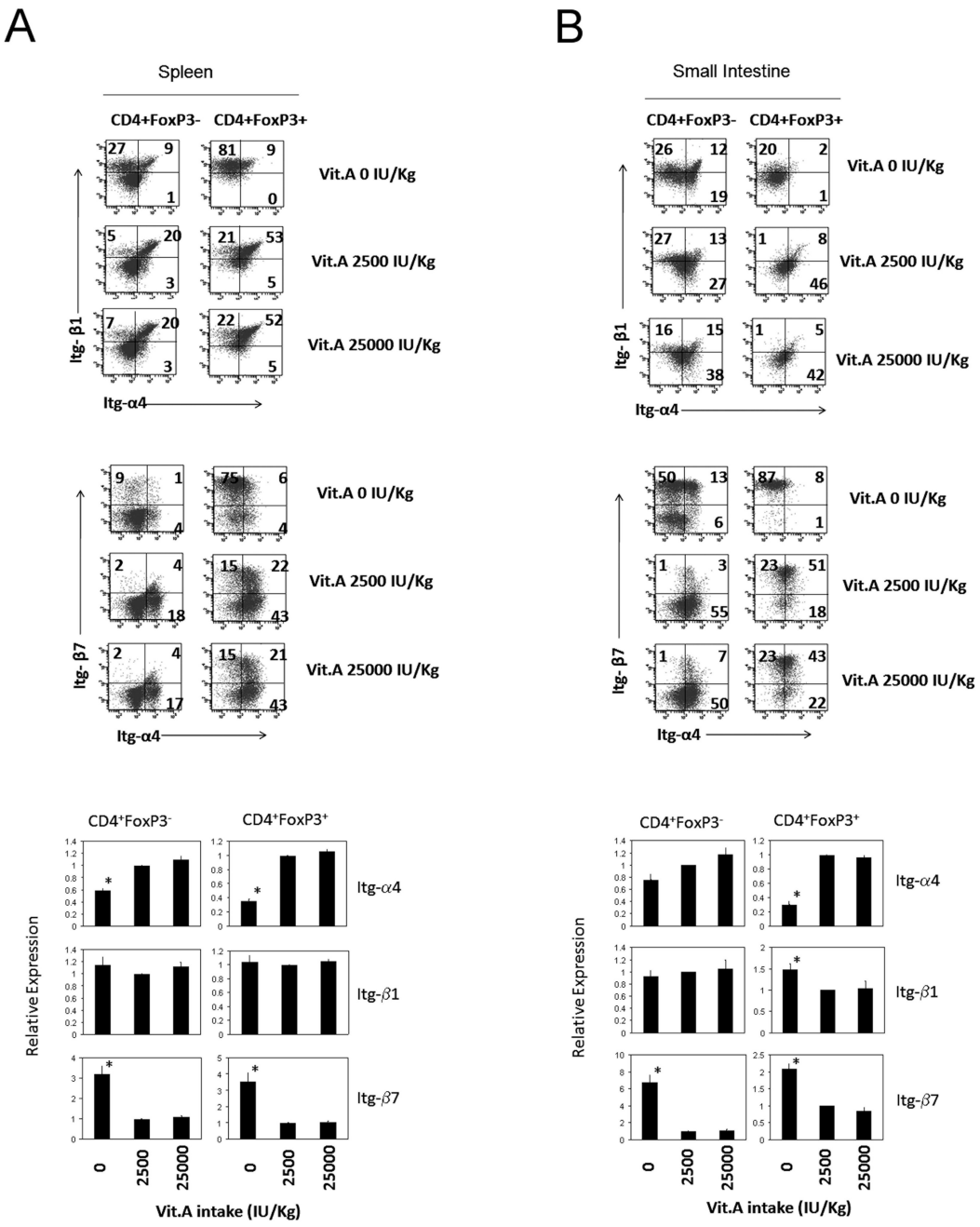


Figure 2. Expression of integrin proteins on the T cell surface in response to a RA gradient

(A) Mouse CD4$^+$ T cells were activated in the absence or presence of Ro41 or RA for 6–7 days and cell surface expression of selected integrins was determined by flow cytometry. TGFβ1 (1 ng/ml) was added to indicated cultures to determine any synergistic effects on induced FoxP3$^+$ and FoxP3$^-$ T cells. (B) Expression of Itg-α4 and Itg-β1 by α4β7$^+$ and α4β7$^-$ CD4$^+$ T cells treated with RA and/or TGFβ1. (C) Effects of various RAR agonists and antagonists on expression of Itg-α4, Itg-αE, Itg-β7 and Itg-β1 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.



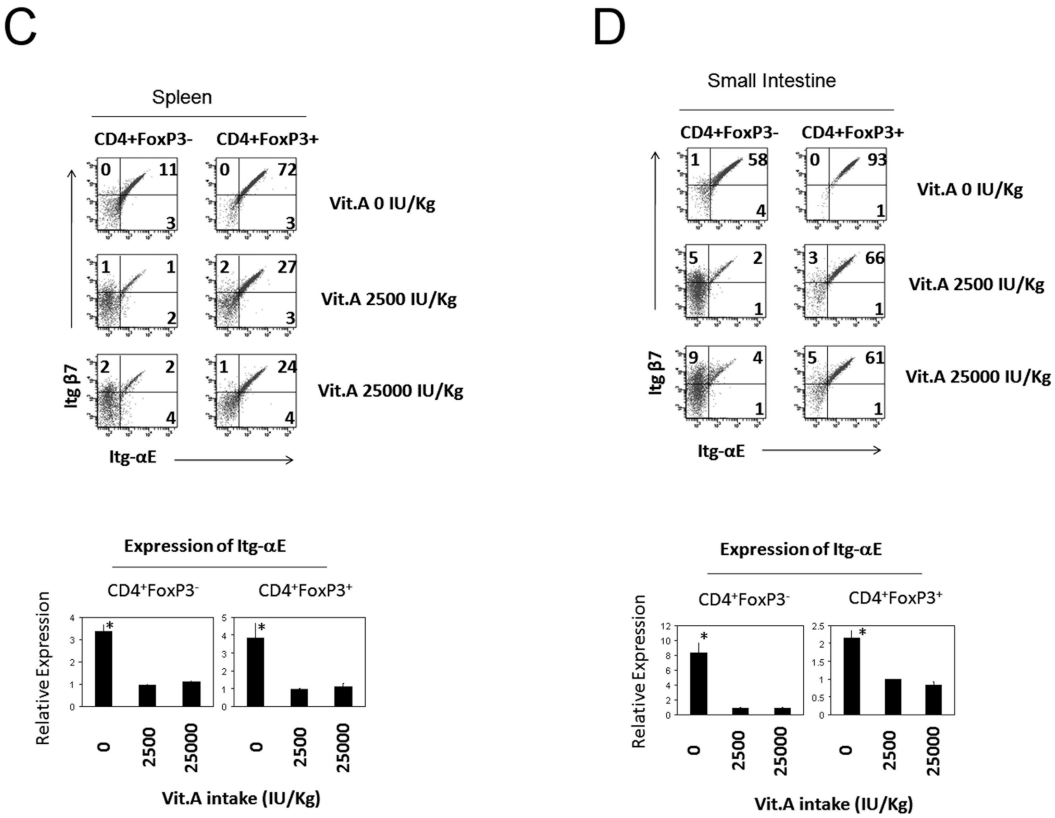
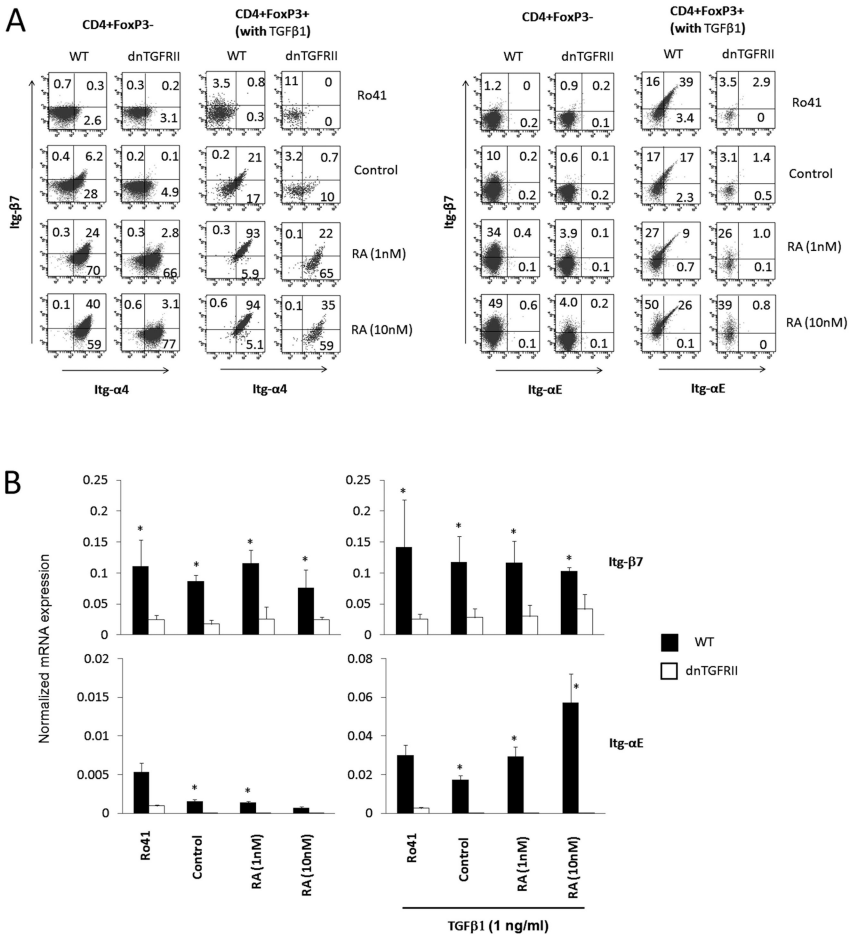


Figure 3. Expression of integrins in vitamin A deficiency

Expression of Itg-α4 and Itg-β7 by spleen (A) and small intestinal lamina propria (B) T cell subsets in vitamin A deficient, normal and high mice. Expression of Itg-αE and Itg-β7 by spleen (C) and small intestinal lamina propria (D) 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 for 12–13 weeks following birth. Representative and combined data (n=4) are shown. *Significant differences from the controls (2,500 U/Kg).



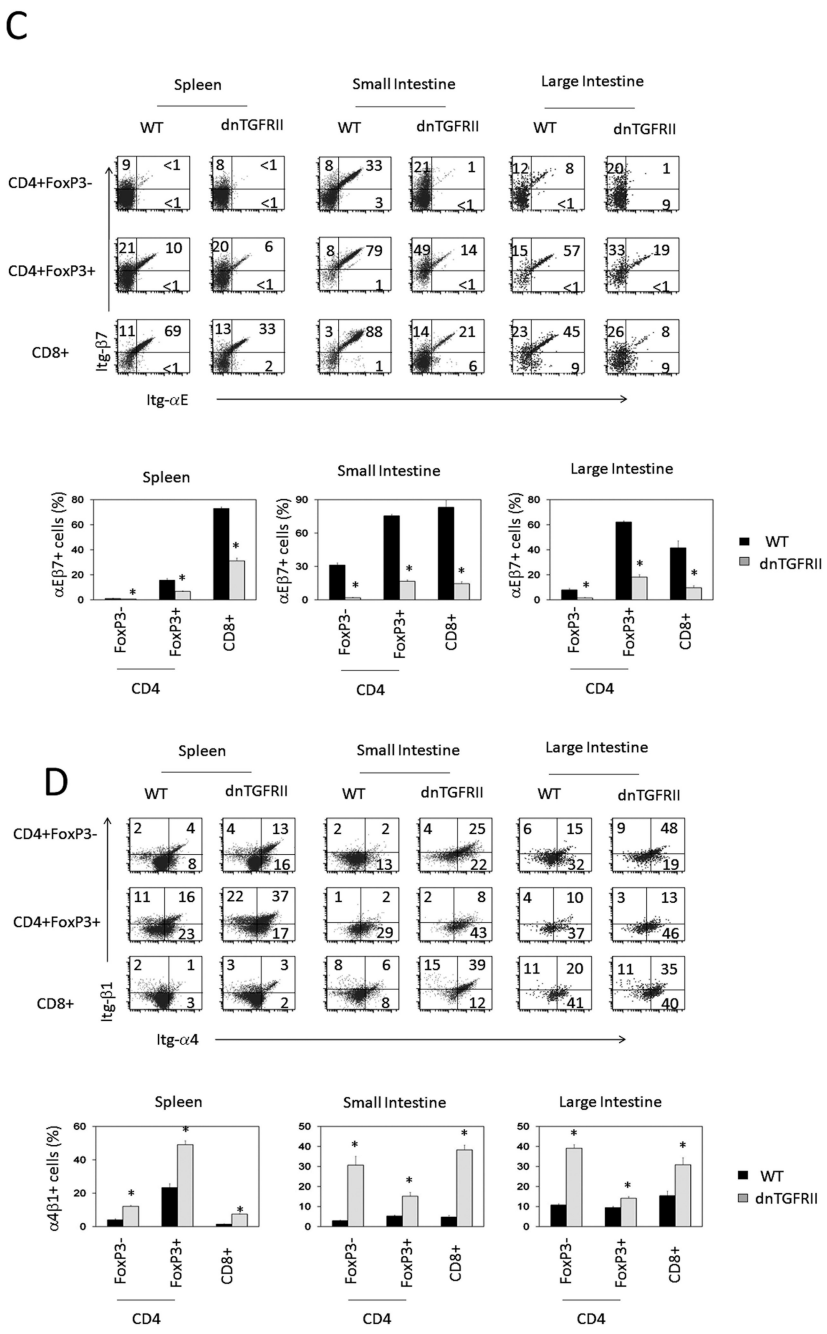
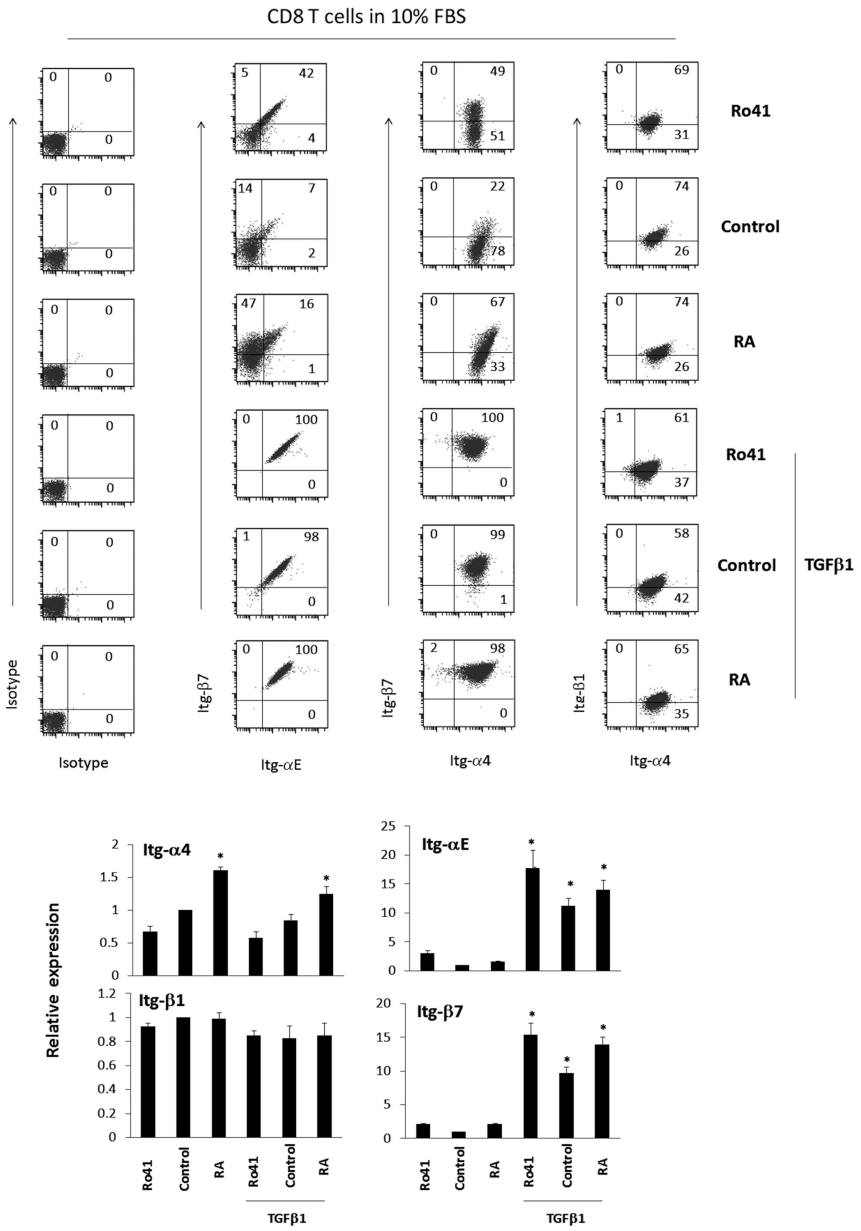


Figure 4. TGFβ1 signal is required for expression of Itg-β7 and Itg-αE

(A) Surface expression of α4β7 and αEβ7 in the presence and absence of TGFβ signal. A RA gradient is made with Ro41, RA (1 nM), and RA (10 nM) in 10% FBS-containing medium. TGFβ1 (1 ng/ml) was added to indicated cultures. Naïve $CD4^+$ T cells, isolated from wild type or dnTGFβRII mice, were cultured for 6–7 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 SEM of 3 independent experiments is shown. (C) Expression of Itg-αE and Itg-β7 by the T cells in dnTGFβRII

mice. (D) Expression of Itg-α4 and Itg-β1 by the T cells in dnTGFβRII mice. The graphs show combined data (% positive cells among each T cell subset) obtained from 3 different mice per group.

A



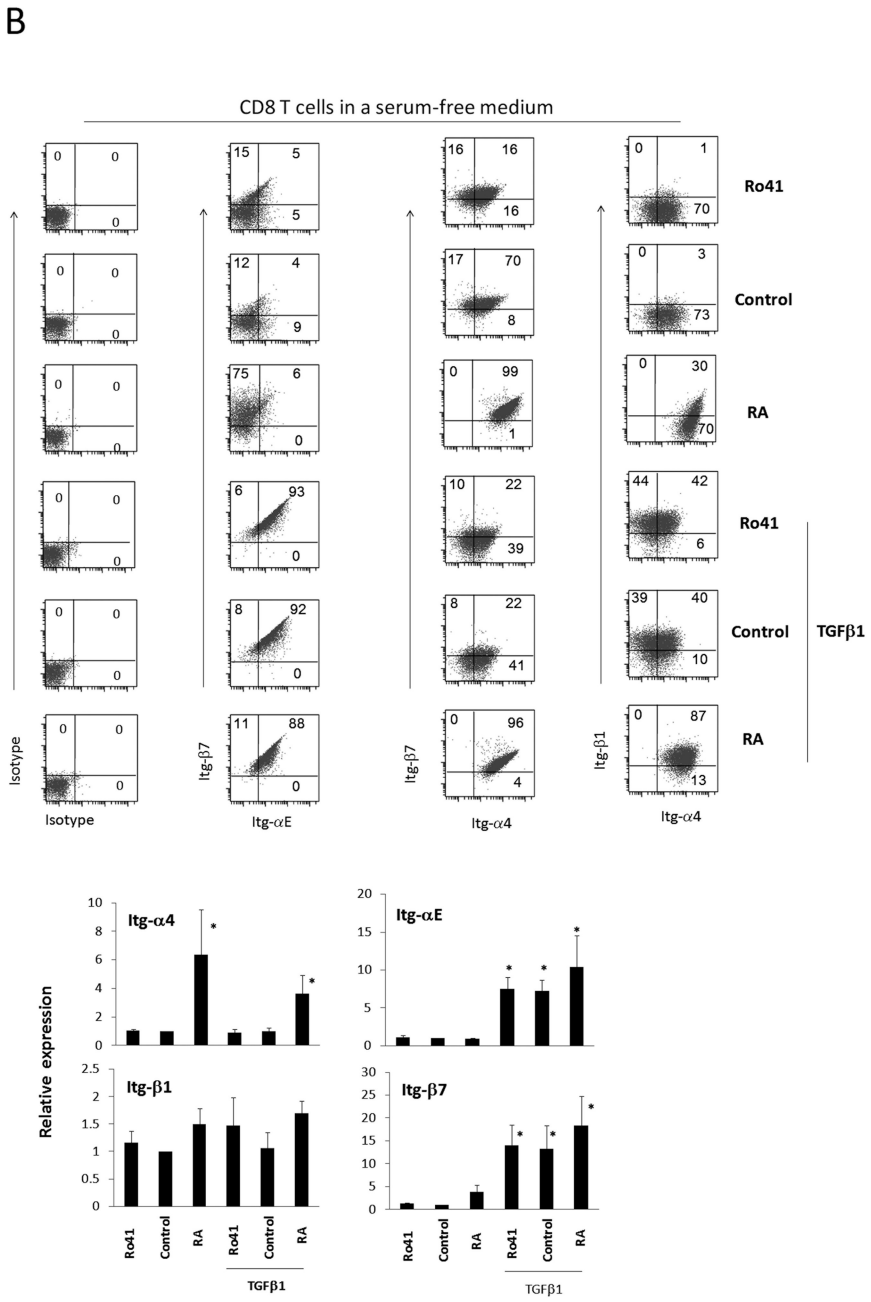


Figure 5. Regulation of integrins on CD8$^+$ T cells by RA and TGFβ1

Mouse CD8$^+$ T cells were activated in the absence or presence of Ro41 or RA for 5–6 days in a 10% FBS-containing medium (A) or a serum free medium (B), 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) 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.

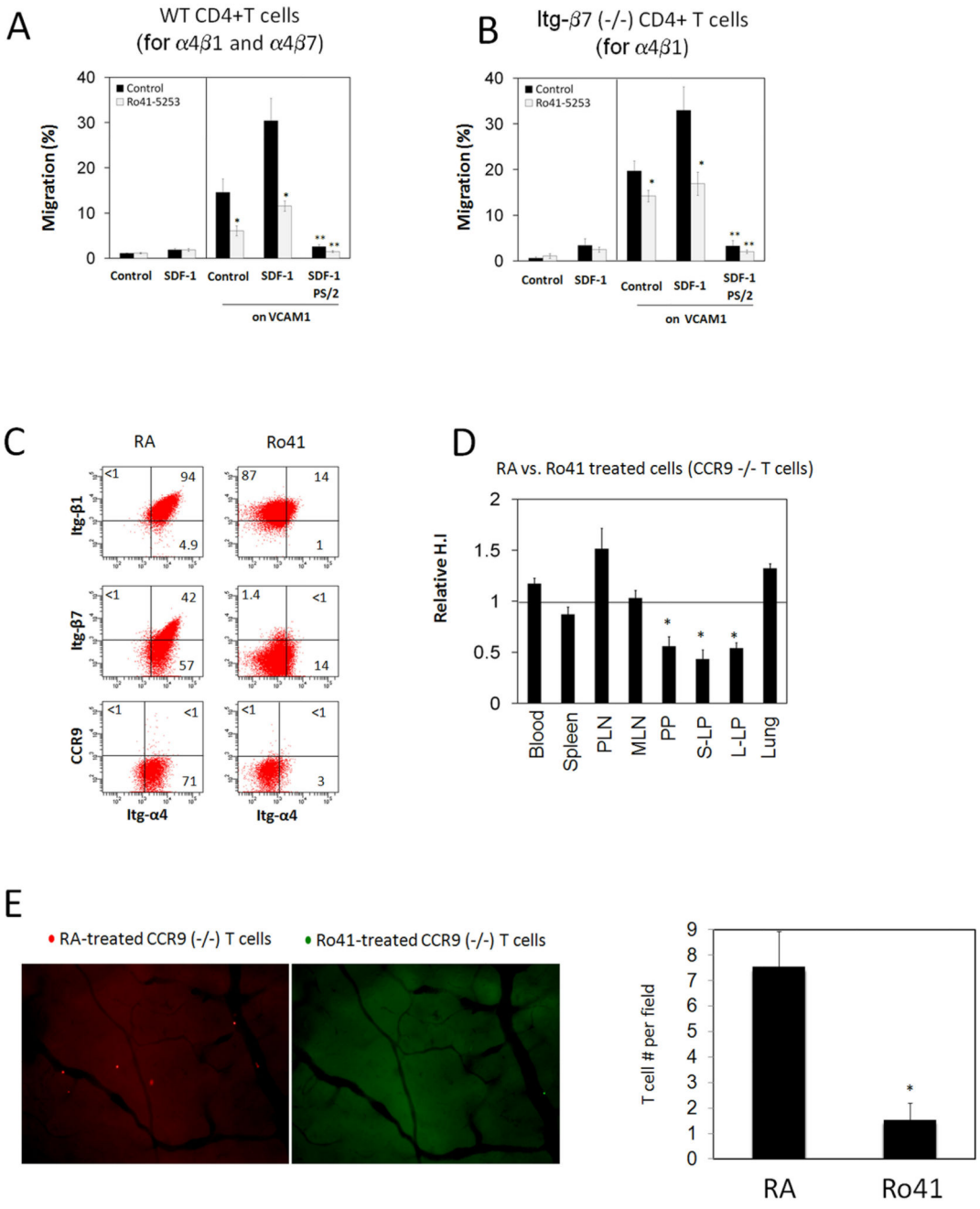


Figure 6. Functional impacts of Itg-α4, induced by low concentrations of RA, on migration of T cells *in vitro* and *in vivo*

(A and B) Migration of Itg-α4-low and normal T cells through VCAM1-coated Transwells in response to SDF-1 or control medium was examined. Wild type T cells can express both α4β1 and α4β7, while Itgβ7 (−/−) T cells express α4β1 but not α4β7. Combined data of three independent experiments are shown. Naïve $CD4^+$ T cells, isolated from wild type (A) or Itgβ7 (−/−) (B) mice were cultured in the presence of control medium or Ro41 to prepare control and Itgα4-low T cells respectively. PS/2, an Itg-α4 blocking monoclonal antibody, was used to block the Itg-α4-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. CCR9 (−/−) naïve $CD4^+$ T cells were cultured in the presence of RA or Ro41 to prepare control and Itg-α4-low T cells. CCR9 (−/−) naïve $CD4^+$ T cells were used to exclude the effect of CCR9 (another trafficking receptor induced by RA) on migration. (D) Migration Itg-α4-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 lower than 1 indicate decreased homing compared to control T cells. Combined data (n=4–6). (E) Intravital microscopy was performed to visualize the migrating Itg-α4-low and control CCR9 (−/−) T cells to Peyer's patches immediately following the T cell transfer. A representative set of data of three independent experiments is shown. Error bars indicate STD of # of cells per field (# cells rolling, arrested, and migrated) in Peyer's patches in ~20 image frames. *Significant decreases in migration.

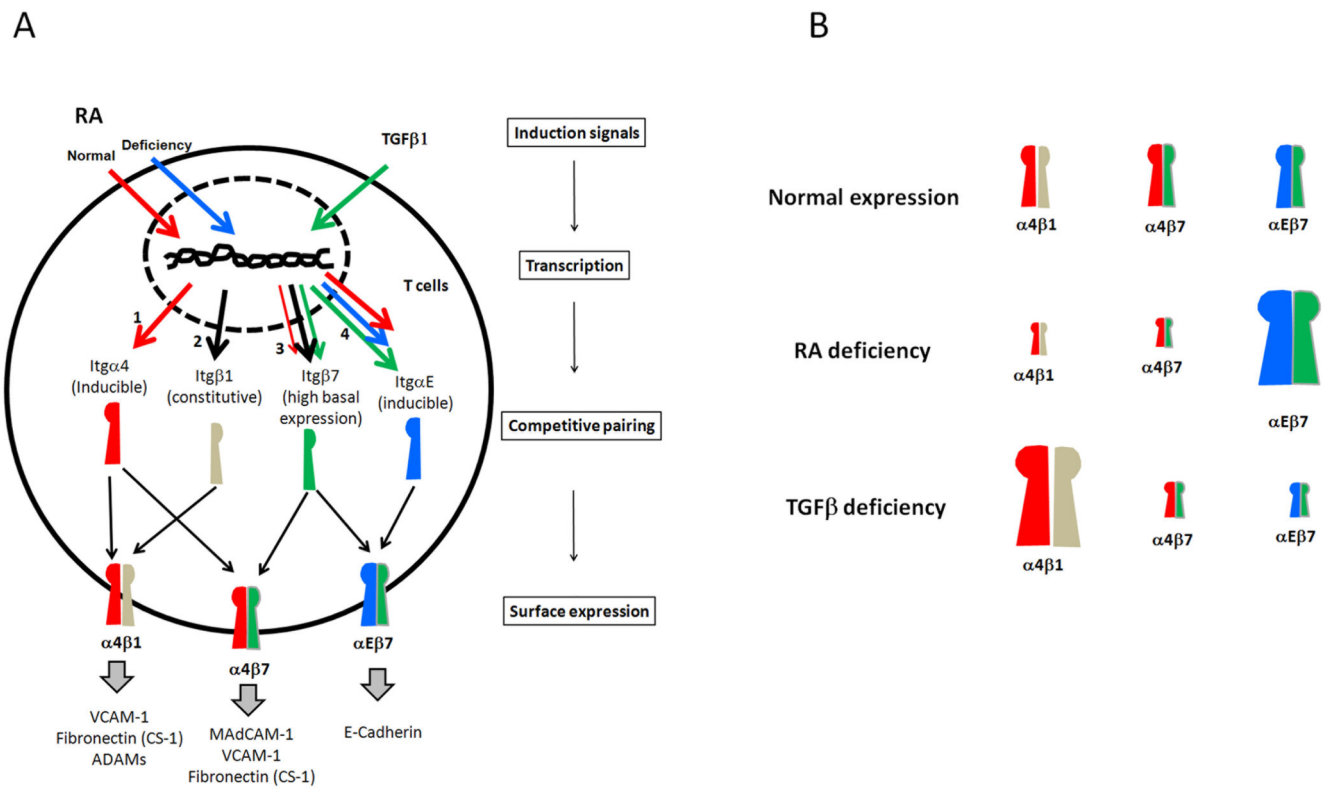


Figure 7. Coordinated regulation of the expression of α4β1, α4β7 and αEβ7 by retinoic acid and TGFβ1 in T cells

(A) RA positively induces Itg-α4 (red lines) but RA paucity induces Itg-αE (blue lines). In contrast, Itg-αE is induced by TGFβ1 (green lines), while Itg-β1 is constitutively expressed by activated T cells. Itg-β7 is constitutively expressed but can be further induced by TGFβ1. Also, RA appears to increase Itg-β7 expression in $CD8^+$ T cells. Because of the heterodimerization requirement, expression of the three integrins is influenced by RA and TGFβ signals in combination. α4β1 can be induced by RA alone, whereas high expression of α4β7 requires both RA and TGFβ1. High expression of αEβ7 requires TGFβ1. In the intestine and other tissues where RA is available at optimal concentrations, high expression of α4β1 and α4β7 by T cells occurs. (B) In vitamin A deficiency, decreases in expression of α4β1 and α4β7 but increases in αEβ7 occur due to decreased Itg-α4 and increased Itg-αE expression. In a TGFβ signal deficiency, α4β1 is over-expressed because the expression of α4β7 (and αEβ7) is suppressed, freeing up Itg-α4 molecules for pairing with Itg-β1. Reciprocally, α4β7 is over-expressed when α4β1 is not expressed due to Itg-β1 deficiency. 40 This regulatory mechanism operates in most T cells including $FoxP3^+$, $CD4^+$, $CD8^+$ T cells. The size of integrins in the diagram signifies the amount of expression.

Table 1

T cell genes regulated by RA at different RA conditions

Naïve CD4$^+$ T cells were cultured in three different conditions containing 10 nM RA (RA), no exogenous RA (Con; low levels of residual RA present in the medium containing 10% FBS), and Ro41 (Ro; a RARα antagonist to block the effect of residual RA on RARα). Expression ratios of genes that are up-or down-regulated in 3 different comparisons are shown. The raw and processed data are deposited at the GEO array data base (www.ncbi.nlm.nih.gov/geo; the accession number is GSE20500).

Name	Affymetrix ID	GenBank ID	RA/Ro	Con/Ro	RA/Con
RA Up-regulated 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_001038839 7	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

Name	Affymetrix ID	GenBank ID	RA/Ro	Con/Ro	RA/Con
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 Down-regulated 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
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

Name	Affymetrix ID	GenBank ID	RA/Ro	Con/Ro	RA/Con
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