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Control of α4β7 Integrin Expression and CD4 T Cell Homing by the β1 Integrin Subunit

Christopher C. DeNucci,* Antonio J. Pagán,† Jason S. Mitchell,* and Yoji Shimizu*

The α4β7 integrin promotes homing of T cells to intestinal sites. The α4 integrin subunit that pairs with β1 integrin can also pair with β7 integrin. In this paper, we show that the preferential pairing of β1 integrin with α4 integrin regulates the expression of α4β7 on T cells. In the absence of β1 integrin, naive mouse CD4 T cells have increased α4β7 expression, resulting in increased adhesion to mucosal addressin cell adhesion molecule-1 and enhanced homing to Peyer’s patches (PP). In a reciprocal manner, overexpression of β1 integrin causes the loss of α4β7 expression and decreased homing to PP. A similar upregulation of β1 integrin and suppression of α4β7 expression occurs rapidly after CD4 T cell activation. β1 integrin thus dominates β7 integrin for α4 integrin pairing, thereby controlling the abundance of unpaired α4 integrin. Increasing the abundance of α4 integrin relative to β1 integrin is critical to retinoic acid-mediated expression of α4β7 integrin during T cell activation. In the absence of β1 integrin, endogenous Ag-specific CD4 T cells uniformly express high levels of α4β7 after *Listeria monocytogenes* infection. The resulting β1-deficient early memory T cells have decreased localization to the bone marrow and enhanced localization to PP after infection. Thus, the preferential association of β1 integrin with α4 integrin suppresses α4β7 integrin expression and regulates the localization of memory CD4 T cells. *The Journal of Immunology, 2010, 184: 2458–2467.*

Integrins are heterodimeric cell surface expressed adhesion molecules composed of noncovalently linked α and β subunits (1). T cells express several integrin family members that are involved in activation, trafficking, and retention in tissue (2, 3). On T cells, the α4 integrin subunit associates with either the β1 subunit, to form α4β1 integrin, or the β7 subunit, to form α4β7 integrin. Both α4β1 and α4β7 are expressed at low levels on naive T cells (4). The β7 integrin subunit can also pair with the αε subunit, which is expressed on naive CD8 T cells (5) and CD4 regulatory T cells (6) but not naive CD4 T cells. The α4 integrins, along with α4β2 (LFA-1), promote recirculation through secondary lymphoid organs at steady state (3, 7). Although α4β1 also localizes to the immunological synapse that forms between a T cell and an APC (8), the in vivo relevance of α4 integrins for T cell activation by APCs remains unclear (9, 10).

During T cell activation, the expression of integrins changes to promote the entry of T cells into nonlymphoid sites. In contrast to low levels of both β1 and β7 integrin on naive CD4 T cells, human memory CD4 T cells express either high levels of α4β1 or high levels of α4β7 integrin (4, 11, 12). This reciprocal high expression of either α4β1 or α4β7 promotes altered trafficking properties based on the site-specific expression of the α4β1 ligand, VCAM-1, and the α4β7 ligand, mucosal addressin cell adhesion molecule-1 (MAdCAM-1). VCAM-1 is expressed at high levels on the vasculature of the bone marrow (BM) (2) and the inflamed brain (13). Thus, α4β1 expression is critical for effector/memory T cell entry into these sites (10, 14). In contrast, MAdCAM-1 is specifically expressed at steady state on the venules of the mesenteric lymph node (mLN) and Peyer’s patches (PP), and becomes highly upregulated on intestinal venules during inflammation (15, 16). Expression of α4β7 on T cells has been associated with preferential trafficking to the intestine (17). The role of α4 integrins play in directing site-specific homing has made them attractive therapeutic targets for treatment of multiple sclerosis and inflammatory bowel disease (18, 19).

Recent studies have identified T cell extrinsic factors that control the expression of α4β7 and the generation of gut homing T cells (20). This work has revealed that retinoic acid (RA) produced by intestinal dendritic cells (DCs) and/or stromal cells specifically promotes expression of α4β7 and CCR9 on T cells (21–23). In contrast, the vitamin D metabolite, 1,25 dihydroxy-VitD₃, suppresses RA-driven induction of α4β7 and CCR9 while enhancing the expression of skin-homing molecules in human T cells (24, 25). These results suggest that the regulation of homing molecules during T cell activation involves the integration of a variety of both positive and negative signals.

The T cell intrinsic factors that regulate the expression of α4 integrins on T cells are not known. As both α4β7 and α4β1 share a common α subunit, we predict that their expression is interrelated. In this study, we show that the loss of β1 integrin on mouse CD4 T cells results in increased α4β7 expression, whereas high-level expression of β1 integrin results in the loss of α4β7 expression. Interestingly, alterations in β7 integrin do not produce reciprocal changes in β1 integrin expression. We demonstrate that β1 integrin regulates the expression of α4β7 expression through preferential pairing with α4 integrin. In the absence of β1 integrin, CD4 T cells aberrantly express high levels of α4β7 in the spleen, resulting in enhanced localization to the PP and reduced maintenance in the BM.
Materials and Methods

Mice

β1 integrin floxed mice (26) were backcrossed to the C57BL/6 background for >14 generations and then crossed with CD4-Cre transgenic mice (27). β3 integrin-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME) (28). Human coxsackie adenovirus receptor (hCAR) transgenic mice (29) were provided by Dr. C. Weaver (University of Alabama-Birmingham). All mice were housed and bred under specific pathogen-free conditions and generally used between the ages of 6–12 wk. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Cell preparation and flow cytometry

Single-cell suspensions were prepared from the spleen, lymph nodes, BM, and PP by mashing through cell strainers. BM cells were flushed from the bursa, hind leg tibia, and femurs using PBS/2% calf serum. PP were dissected from the small intestine, mechanically disrupted, and digested in HBSS/HEPES/10% calf serum and 400 units/mL collagenase D (Roche, Basel, Switzerland) for 30–45 min at 37°C. Purified T cells were obtained using negative selection by depleting cells expressing B220, I-A<sup>b</sup>, CD16/32 (Abs all from eBioscience, San Diego, CA) using MACS LS columns (Miltenyi Biotec, Auburn, CA). For naïve CD4 T cell purification, anti-CD8a and anti-CD44 (both from eBioscience) were also added. For flow cytometry, 1–5 × 10<sup>6</sup> cells were stained in HBSS/0.2% sodium azide/2% calf serum (FACS buffer) for 20–30 min at 4°C. Samples were collected on a BD LSRII (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Intracellular integrin staining

After CD4 T cell purification (~95% purity), cells were resuspended in 0.01% pronase (EMD Chemicals, Darmstadt, Germany) in PBS for 30–45 min at 37°C to nonspecifically remove cell surface proteins. Samples were washed twice with FACS buffer supplemented with 10% calf serum and then fixed with 2% paraformaldehyde for 25 min at room temperature. Fixed samples were stained once prior to permeabilization with the same Abs to be used for intracellular staining. Cells were permeabilized using 0.2% Triton-X 100 in PBS/0.2% BSA for 7–9 min at room temperature. Staining for intracellular integrin was performed for 20–30 min at room temperature with anti-β1 integrin (HMβ1.1-APC) (Biolegend, San Diego, CA) and anti-β3 integrin (M293-PE) (BD Bioscience) Abs.

Adhesion assays

Adhesion assays were performed as previously described (30). Purified T cells labeled with calcine-AM (Invitrogen, Carlsbad, CA) were added to wells coated with recombinant mouse VCAM-1 (0.6 μg/mL), MadCAM-1 (6 μg/mL), or ICAM-1 (6 μg/mL) (R&D Systems, Minneapolis, MN). Cells were incubated with anti-CD3ε (2C11) (eBioscience) and integrin blocking anti-β1 (Ha2/5, 10 μg/mL), anti-β2 (Fib27, 10 μg/mL), or anti-αL (M17/4, 10 μg/mL) (all from BD Bioscience) Abs for 15-min binding time at 4°C, followed by a 15-min stimulation at 37°C. Wells were washed and the percentage of adherent cells was determined by comparing well-fluorescence postwash to prewash. For adhesion assays with adeno-virus transduced cells, flow cytometry was used to quantify percentage of adherent Thy1.1 high CD4+ cells after stimulation with 50 ng/mL PMA.

Short-term in vivo cohoming assays

Lymph node cells from β1<sup>LacZ</sup> (CD4 Cre<sup>−</sup>) and β3<sup>LacZ</sup> (CD4 Cre<sup>−</sup>) mice (or Thy.1 and β1 virus transduced T cells) were labeled with either Cell Tracker Green CMFDA (0.25 μM) or Cell Tracker Orange CMTMR (2 μM) (Invitrogen). Reversing these colors did not alter the experimental outcome. Cells were then equally mixed and i.v. injected at 5–10 × 10<sup>6</sup> cells/mouse. An aliquot of the mixture was taken prior to injection to serve as the input control. At 2 or 24 h postinjection, the indicated organs were harvested. Transferred cells were identified by intravital labels and CD4<sup>+</sup> staining by flow cytometry. For the adeno-virus transduced T cell cohoming assays, Thy1.1 staining was additionally used to identify transduced cells. The homing index was calculated as previously described (31).

Mixed BM chimeras

Mixed BM chimeras were generated by mixing T cell-depleted BM cells from β1<sup>LacZ</sup> (CD4 Cre<sup>−</sup>) (CD45.1/45.2) and β3<sup>LacZ</sup> (CD4 Cre<sup>−</sup>) (CD45.2) mice and transferring 5–10 × 10<sup>6</sup> cells i.v. into irradiated (1000 cGy) B6.SJL (CD45.1) recipients. Tissues were harvested 8–15 wks post-BM transplant and single-cell suspensions were FACS stained for CD3e, CD4, CD8a, B220, CD45.1, CD45.2, and CD44. The percentage of CD45.2<sup>+</sup> and CD45.1/45.2<sup>+</sup> CD4<sup>+</sup> cells recovered from each organ was normalized to the percentage recovered from the spleen.

In vitro stimulation assay

Purified naïve T cells labeled with 5 μM CFSE were stimulated with plate-bound anti-CD3ε (3 μg/mL)/anti-CD28 (3 μg/mL) and maintained in T cell proliferation media (TCPM) (RPMI medium 1640 with 1-glutamine and 25 mM HEPES [Invitrogen] supplemented with 10% FBS [Atlanta Biologicals, Lawrenceville, GA], 100 mM sodium pyruvate, penicillin/streptomycin, and 5 μM 2-ME). Cells were harvested at 3 d after plating and FACS stained for CD4, β1 integrin (HMβ1.1-APC) (Biolegend), integrin (R1-2-PE) (eBioscience), and αβ integrin (DATK32-Bio, SA-PECY7) (eBioscience). For the RA treatment experiments, RA (R6265, Sigma-Aldrich, St. Louis, MO) was added at 1000 nM in DMSO to the TCPM at the start of stimulation and cells were harvested after 2 d. DMSO carrier alone was added to “No RA” samples.

Adenovirus production and transduction

Murine β1 integrin (mCD29) and α4 integrin (mCD49d) cDNA clones (Open Biosystems, Huntsville, AL) were subcloned by PCR into the pENTR-UP-IT vector using Sall and BamHI restriction sites. The production of adenovirus and transduction of T cells was performed as previously described (29, 32, 33). The isolated lymph node cells from hCAR expressing mice were transduced with either Thy1.1 control or integrin virus and cultured for 2–3 d in TCPM plus 10 ng/ml mouse IL-7 (R&D Systems) then FACS stained for CD4, β1 integrin, α4 integrin, and αβ integrin as above.

Quantitative real-time RT-PCR

The 1–3 × 10<sup>6</sup> purified CD4 T cells were homogenized using QIAshredder, and RNA was isolated with an RNasey kit (Qiagen, Valencia, CA). cDNA was produced from equivalent amounts of RNA with the Superscript III Platinum Two-Step qRT-PCR Kit (Invitrogen) and the PCR products were amplified with the Fast Start SYBR Green Master Mix (Roche). Samples were detected on ABI PRISM 7000 (Applied Biosystems, Foster City, CA). Hypoxanthine phosphoribosyltransferase was used to normalize samples and the comparative Ct method was used to quantitate relative mRNA expression (34). Primers were designed using Primer Express (Applied Biosystems). The primer sets used were as follows: hypoxanthine phosphoribosyltransferase (forward primer 5′-CTTCTCCTCTACGACCGCTTT-3′, reverse primer 5′-ACCTGGTTCATACATGCTTCA-3′); α4 integrin (forward primer 5′-AATTGGACAACTGCTTGGGACCA-3′, reverse primer 5′-TGGTCTGATATCCATAACAAATGGGT-3′); β1 integrin (forward primer 5′-AATTCGCAAATTCTTCCGGAGAA-3′, reverse primer 5′-TCCCTGACATCATCCTATGCT-3′); β2 integrin (forward primer 5′-GATAACATGTCACAGAGGAGGACATGT-3′, reverse primer 5′-CCGAAAGATGGCGCTGT-3′); β3 integrin (forward primer 5′-TGCGTCATGATGGCTTCA-3′, reverse primer 5′-CCGGTCCCTCCACGACCGCTTTACA-3′).

Listeria infections and peptide:MH C class II-tetramer enrichments

Mice were infected i.v. with 1 × 10<sup>7</sup> CFUs of Acta-deficient Listeria monocytogenes expressing 2W1S (A Lm-2W1S) (35). 2W1S:1-α4 tetramer production, staining, and T cell enrichment were completed as previously described (36, 37). The enriched fraction of cells was FACS stained with an Ab mixture for CD3ε, CD4, CD8ε, and CD44. B220, CD11b, CD11c, and F4/80 were used in a dump gate to exclude cells binding tetramer nonspecifically. CD11b and F4/80 were not used in the dump gate for the PP. Some samples were additionally labeled with anti-β7 integrin (Fib504-PE) (Biolegend) alone or anti-β1 integrin (HMβ1.1-PE) (eBioscience) and anti-αβ integrin (DATK32-PE) (eBioscience). Total tetramer positive events were enumerated using CALTAG Counting Beads (Invitrogen). Assuming that only 20% of total body BM cells are resident in the hindlimb (38), the number of tetramer positive cells in the BM was multiplied by five to obtain total BM cell numbers (14). The steady-state percentage of cells in the BM and PP was calculated on a per mouse basis by dividing the number of 2W1S-specific CD4<sup>+</sup> T cells recovered from the tissues by the number of cells from the spleen of the same mouse.

Statistical analysis

All statistical analysis was performed using GraphPad Prism software 5.0 (La Jolla, CA). Two-tailed t test or one-way ANOVA, followed by Tukey’s multiple comparison test were used to assess significance.
Control of α4β7 integrin expression by β1 integrin

Results

Loss of β1 integrin on CD4 T cells results in increased surface expression of α4β7

To evaluate the function of β1 integrin on T cells, we crossed mice with a floxed β1 integrin gene (β1fl/fl) (26) with transgenic mice expressing Cre recombinase under the control of the CD4 promoter (CD4-Cre) (27). In β1fl/fl CD4-Cre+ mice (β1−/− mice), there is a complete loss of β1 integrin on the majority of CD4 T cells collected from peripheral lymph nodes when compared with β1fl/wt CD4-Cre+ controls (Fig. 1A). However, the surface expression of α4 integrin, a major pairing partner for β1 integrin, remained unchanged on β1−/− CD4 T cells. As intracellular α/β subunit pairing is required for cell surface integrin expression, this suggests that enhanced cell surface expression of β7 integrin, the other known pairing partner for α4 integrin, is responsible for maintaining α4 integrin expression on β1−/− T cells. Using an Ab that specifically recognizes the α4β7 integrin, we found that α4β7 was significantly elevated on β1−/− CD4 T cells (Fig. 1A, 1B). The loss of β1 integrin did not result in changes in mRNA levels for either the α4 integrin subunit or the β7 integrin subunit (Fig. 1C), consistent with the idea that enhanced α4β7 expression is occurring at the level of α/β subunit pairing. The enhanced surface expression of α4β7 observed on β1−/− T cells suggests that wild type (wt) CD4 T cells may contain intracellular stores of β7 integrin that are capable of pairing with unpaired α4 integrin. We used pronase to remove cell surface proteins from naive CD4 T cells, followed by permeabilization and staining with anti-integrin Abs to assess expression of intracellular integrin subunits. Wt CD4 T cells had clearly detectable levels of intracellular β7 integrin subunit (Fig. 1D).

In contrast to the results obtained with loss of β1 integrin expression, CD4 T cells from β7 integrin-deficient mice showed no increase in β1 integrin and did not maintain α4 integrin expression at wt level (Fig. 1E). These results predict that the majority of β1 integrin is cell surface expressed, leaving excess unpaired α4 integrin without a pairing partner for cell surface expression when β7 integrin is lost. This is in agreement with the minimal levels of intracellular β1 integrin observed in wt CD4 T cells (Fig. 1D). Overall, these results suggest that through competition for α4 integrin pairing, β1 integrin expression limits the amount of α4β7 that is expressed on the surface of naive CD4 T cells.

β1−/− CD4 T cells have altered adhesion to α4 integrin ligands and trafficking to the BM and PP

To determine the functional consequence of loss of β1 integrin, we analyzed the adhesion of control and β1−/− T cells to VCAM-1 and MAdCAM-1. Control T cells showed low basal levels of adhesion to VCAM-1 that were dramatically enhanced by TCR stimulation (Fig. 2A). Adhesion to VCAM-1 was inhibited by a blocking anti-β1 integrin Ab. In contrast, β1−/− T cells did not adhere to VCAM-1 under any tested stimulation condition. β7-deficient CD4 T cells had normal adhesion to VCAM-1, further supporting that the loss of β7 integrin does not result in enhanced expression or function of α4β1 integrin (Supplemental Fig. 1). The elevated expression of α4β7 on β1−/− T cells did result in enhanced adhesion to MAdCAM-1 compared with control T cells (Fig. 2A). Adhesion of both control and β1−/− T cells to MAdCAM-1 was inhibited by a β7 integrin-specific Ab. The loss of β1 integrin on CD4 T cells did not alter mRNA transcript for β2 integrin, expression of LFA-1 (αLβ2), or adhesion to ICAM-1 (Fig. 1C, Supplemental Fig. 2).

We next performed short-term in vivo cohomming assays to determine how the loss of β1 integrin expression alters CD4 T cell homing. Control and β1−/− CD4 T cells were differentially labeled with intravitral dyes, mixed in equal numbers, and transferred into recipient mice. At 2 h, equivalent numbers of control and β1−/− transferred CD4 T cells were recovered from the spleen, inguinal lymph nodes (iLNs), and mLNs (Fig. 2B). In contrast, a significantly higher number of β1−/− CD4 T cells were isolated from the PP of the small intestine. This result is consistent with the elevated levels of α4β7 integrin on β1−/− T cells and the high levels of MAdCAM-1 expressed on venules in the PP. Expression of the gut-homing chemokine receptor CCR9 was not different between β1−/− and control CD4 T cells (Supplemental Fig. 3). Fewer β1−/− CD4 T cells were recovered from the BM, an area rich in the β1 integrin ligand VCAM-1. Transferred CD4 T cell numbers were also examined at 24 h to assess retention (14) at these sites. Differences in the localization of control and β1−/− T cells to the PP and BM that were observed at 2 h were maintained at 24 h (Fig. 2B).

To examine the long-term steady-state distribution of naive β1−/− CD4 T cells, we generated mixed BM chimeras. The percentage of control and β1−/− CD4 T cells in various tissues was examined 8–15 wk after marrow transplant. No gross defect in CD4 T cell...
We next examined changes in aβ4 integrin ligands and trafficking to the BM and PP. A. Adhesion of CD4-Cre+ (wt) and CD4-Cre− (β1−/−) purified T cells to plate bound VCAM-1 or MadCAM-1 after stimulation with soluble anti-CD3e (2C11) Ab. Integrin blocking Abs were used to demonstrate the specificity of T cell-ligand interactions. The bars represent mean values from four replicates in one representative experiment of 3–4, B, β1−/− wt and β1+β2 β1−/− CD4 T cells were differentially labeled, equally mixed, and transferred into recipient mice. Recipient organs were harvested at 2 and 24 h posttransfer and the ratio of CD4 transferred cells recovered was normalized to the transfer ratio from the mixed sample prior to injection (input). Values >1 indicate that β1−/− CD4 T cells were present in higher numbers than wt. Bars represent mean with SEM (n = 3–5 mice from two independent experiments). C. Percentage of β1−/− CD4+CD44high T cells recovered from the lymphoid organs of wt β1−/− mixed chimeric mice. All samples are normalized to the percentage of splenic β1−/− CD4+CD44high T cells, which was set to 50%. The values represent altered steady-state distribution of β1−/− CD4+CD44high T cells compared with the percentage recovered in the spleen. All experimental groups were compared against input or normalized spleen, ns >0.05. *p = 0.01–0.05; ***p = 0.001–0.01; ****p > 0.001, one-way ANOVA, followed by Tukey’s multiple comparison test.

Elevated levels of β1 integrin results in loss of α4β7 expression and function

After T cell activation, the induction of β1 integrin expression directly correlates with suppression of α4β7 expression. To determine whether high expression of β1 integrin is sufficient to suppress α4β7 expression, we overexpressed β1 integrin in naive CD4 T cells. We used recombinant adenovirus expressing a Thy1.1 expression marker and transduced resting naive CD4 T cells isolated from transgenic mice expressing the hCAR (29, 33). Nontransduced cells (no virus) have no Thy1.1 expression and express β1 integrin, α4 integrin, and α4β7 integrin at wt levels (Fig. 4A). Transduction of CD4 T cells with a Thy1.1 control virus did not alter the expression of β1 integrin, α4 integrin, or α4β7 integrin regardless of the level of Thy1.1 expression. Strikingly, CD4 T cells transduced with adenovirus expressing Thy1.1 and β1 integrin exhibited a dramatic loss of α4β7 integrin expression that correlated with increasing β1 integrin expression (Fig. 4A). This finding replicates the alterations in β1 integrin and α4β7 integrin expression observed after anti-CD3/CD28 stimulation of wt CD4 T cells (Fig. 3A). Overexpression of β1 integrin did not alter the level of the α4 integrin on the T cell surface. Unlike wt T cells (Fig. 1D), CD4 T cells overexpressing β1 integrin contained clearly detectable levels of intracellular β1 integrin (Fig. 4B). This indicates that not all exogenously expressed β1 integrin is able to be surface expressed. The amount of intracellular β7 integrin was not increased by overexpression of the β1 integrin subunit (Figs. 1D, 4B). Although β1 integrin mRNA levels were elevated ~20-fold in T cells overexpressing β1 integrin, mRNA levels for α4, β7, and β2 integrin were unaltered (Fig. 4C). These results indicate that β1 integrin subunit outcompetes β7 integrin for α4 integrin pairing at the protein level, resulting in the suppression of α4β7 expression.

In vitro adhesion assays demonstrated that CD4 T cells overexpressing β1 integrin exhibited enhanced activation-dependent
adhesion to VCAM-1 and reduced adhesion to MAdCAM-1 when compared with control T cells (Fig. 4D). In short-term in vivo cohoming assays, overexpression of β1 integrin did not alter CD4 T cell localization to the spleen or iLN at both time points examined (Fig. 4E). However, localization of CD4 T cells overexpressing β1 integrin to the PP was reduced and localization to the BM was enhanced at 2 h and even more dramatically at 24 h posttransfer. A reduced number of T cells overexpressing β1 integrin were also observed in the mLNs at both time points, but this difference was not statistically significant.

Abundance of α4 integrin determines expression of α4β7

Our findings predict that high β1 integrin expression in T cells suppresses α4β7 integrin expression via competition for pairing with the α4 subunit. To create a situation in which the amount of the α4 integrin expressed in T cells is not limiting, we overexpressed α4 integrin.
integrin expression with RA treatment was accompanied by an increase in 
integrin expression compared with CD4 T cells stimulated without RA (Fig. 6A). RA treatment resulted in enhanced integrin staining on Thy1.1 control and α4 virus transduced CD4 T cells. Light gray histograms represent appropriate Iso control staining. B, Representative FACS histograms showing α4, α4β7, and β1 integrin staining on Thy1.1 control and α4 virus cotransduced CD4 T cells. Light gray histograms represent appropriate Iso control staining. C, Comparison of relative β1, α4, and β7 integrin levels between α4 virus and Thy1.1 control transduced CD4 T cells. Real-time RT-PCR data presented as fold change between groups based on the comparative CT method. Bars represent the mean with SEM (n = 3).

Abundance of α4 integrin controls α4β7 integrin expression. A, Representative FACS histograms showing α4, α4β7, and β1 integrin staining on Thy1.1 control and α4 virus transduced CD4 T cells. Light gray histograms represent appropriate Iso control staining. B, Representative FACS histograms showing α4, α4β7, and β1 integrin staining on Thy1.1 control, β1 virus, and β1 and α4 virus cotransduced CD4 T cells. Light gray histograms represent appropriate Iso control staining. C, Comparison of relative β1, α4, and β7 integrin levels between α4 virus and Thy1.1 control transduced CD4 T cells. Real-time RT-PCR data presented as fold change between groups based on the comparative CT method. Bars represent the mean with SEM (n = 3).

The presence of RA during T cell activation promotes increased expression of α4β7 integrin (21). To determine whether RA-induced α4β7 expression is mediated by suppression of β1 integrin or enhancement of α4 integrin, we stimulated naive CD4 T cells with anti-CD3/CD28 Abs for 2 d in the presence of RA. RA treatment resulted in enhanced α4 integrin expression compared with CD4 T cells stimulated without RA (Fig. 6A). The increased α4 integrin expression with RA treatment was accompanied by an ∼4-fold increase in α4 integrin mRNA levels, whereas mRNA levels for β1 and β7 integrin remained unchanged (Fig. 6B). CD3/CD28 stimulation resulted in increased β1 integrin expression that was little affected by the addition of RA (Fig. 6C). In contrast, ∼20% of the RA-treated CD4 T cells at 48 h after stimulation expressed high levels of α4β7 (Fig. 6C). Thus, RA-induced α4β7 integrin expression is likely mediated by upregulation of α4 integrin.

To directly test the ability of β1 integrin to suppress α4β7 integrin upregulation after RA treatment, we stimulated CD4 T cells overexpressing β1 integrin. CD4 T cells transduced with control adenovirus demonstrated similar upregulation of α4β7 as wt cells after stimulation for 2 d (Fig. 6D). In contrast, activated T cells transduced with β1 integrin adenovirus demonstrated no induction of α4β7 expression in the presence of RA. This mirrors our findings with coexpression of α4 and β1 integrin in naive CD4 T cells (Fig. 5B) and demonstrates that high abundance of β1 integrin can suppress RA-induced enhancement in α4β7. These results highlight the importance of α4 and β1 integrin stoichiometry in the control of α4β7 expression.

Ag-specific activation of polyclonal endogenous β1+/− CD4 T cells results in enhanced α4β7

To determine whether β1 integrin expression is critical for regulating α4β7 expression after Ag challenge in vivo, we i.v. infected control and β1+/− mice with an ActA-deficient strain of L. monocytogenes.
expressing the 2W1S variant of peptide 52–68 from the I-E α-chain (A′ Lm-2W1S) (35, 39). At various time points after challenge, the number of 2W1S-specific CD4 T cells was determined using recently described peptide/I-A α-HMC class II tetramer-based enrichment and flow cytometry gating approaches (Supplemental Fig. 6) (36, 37).

Endogenous populations of naive 2W1S-specific CD4 T cells from the spleens of uninfected control and β1−/− mice were equivalent in number (n = 7, wt: 238 ± 29; β1−/−: 234 ± 39 [mean ± SEM]) (Fig. 7A). Infection of control and β1−/− mice with A′ Lm-2W1S resulted in a comparable expansion, contraction, and long-term maintenance of 2W1S-specific CD4 T cells in the spleen out to 120 d (Fig. 7A). However, the expression of α4 integrins on these activated T cells was dramatically different. At the peak of the response (day 5), a surprisingly high percentage of 2W1S-specific control CD4 T cells (50 ± 3%, n = 4) in the spleen expressed high levels of β7 integrin (Fig. 7B). By day 18, the majority of 2W1S-specific CD4 T cells remaining in the spleen had low β7 integrin compared with naive CD4 T cells, but a population of α4β7-high cells (8 ± 2%, n = 5) was maintained. These control 2W1S-specific CD4 T cells demonstrated a reciprocal relationship between β1 integrin and α4β7 integrin expression (Fig. 7C). This population of α4β7-high cells resembles a circulating population of gut homing memory CD4 T cells previously identified in humans that is β1 integrin low and α4β7-high (4, 11). This β7-high T cell population was maintained long-term and represented ~15% of the total splenic population by 60 d postinfection. In contrast, 2W1S-specific CD4 T cells from the spleens of β1−/− mice became uniformly β7-high (98 ± 0.2% n = 4) rapidly after infection and maintained high level expression of β7 integrin out to day 120 (Fig. 7B, 7C). Thus, the vast majority of 2W1S-specific β1−/− CD4 T cells in the spleen resembled the small population of α4β7-high CD4 T cells observed in control mice.

**Altered maintenance of β1−/− early memory CD4 T cells in the BM and PP**

To determine whether loss of β1 integrin and the resulting aberrant upregulation of α4β7 results in altered early memory CD4 T cell localization, we assessed the number of 2W1S-specific T cells in the BM and PP of control and β1−/− mice 18 d after A′ Lm-2W1S infection. This is a time point where we observed similar numbers of 2W1S-specific CD4 T cells in the spleen (Fig. 7A). Yet, in the BM there was a reduced number of 2W1S-specific CD4 T cells in infected β1−/− mice compared with control mice (Fig. 8A, 8B). In contrast, there were increased numbers of 2W1S-specific CD4 T cells in the PP of infected β1−/− mice compared with control mice.

**FIGURE 7.** Polyclonal endogenous β1−/− CD4 T cells in the spleen have enhanced α4β7 expression after i.v. infection. A, Peptide:MHC class II tetramer enrichment was used to quantify the number of endogenous 2W1S-specific CD4 T cells in control β1+/+ (wt) and β1−/− mice splenocytes after A′ Lm-2W1S. Time 0 represents the number of 2W1S-specific recovered from naive wt or β1−/− mice. Symbols represent mean with SEM (n = 4–11 mice/time point, except wt day 120, n = 2). B, Representative histograms of β7 integrin expression on splenic 2W1S-specific CD4 T cells from wt and β1−/− mice. The table indicates mean percentage (± SEM) of β7-high splenic 2W1S-specific CD4 T cells (n = 2–5 mice). C, Representative staining of spleen CD4+ T cells from wt and β1−/− mice 18 d postinfection. β1 integrin versus α4β7 integrin expression is shown on the gated CD44high 2W1S-I-A β tetramer+ cells.

**FIGURE 8.** β1 integrin-deficient early memory CD4 T cells have enhanced localization to the PP and reduced maintenance in the BM. A, Representative 2W1S-I-A β tetramer staining of CD4+ T cells isolated from the BM and PP 18 d after i.v. infection with A′ Lm-2W1S. The bold numbers represent the number of 2W1S-specific CD4 T cells isolated from BM and PP 18 d after A′ Lm-2W1S. The bold numbers below the gate are the total calculated 2W1S-specific CD4 T cells recovered from each sample. B, Individual replicates of 2W1S-specific CD4 T cell numbers recovered from the BM and PP 18 d postinfection. Line represents mean value. *p < 0.02, two-tailed unpaired t test. C, The steady-state percentage represents the number of 2W1S-specific CD4 T cells recovered in the BM or PP compared with the spleen of the same mouse. Bars represent mean with SEM (n = 3–7 mice). *p < 0.04; **p < 0.006, two-tailed unpaired t test.
mice. All the 2W1S-specific CD4 T cells in the BM and PP expressed high levels of CD44, a marker of Ag experience. As a measure of the steady-state maintenance of Ag-specific CD4 T cells in the BM and PP, we also analyzed the percentage of 2W1S-specific CD4 T cells in these tissues relative to the number of 2W1S-specific CD4 T cells in the spleen. This analysis revealed that relative to the spleen, ∼8% of the 2W1S-specific CD4 T cells were found in the BM and only ∼0.1% were found in the PP postinfection of control mice (Fig. 8C). In contrast, postinfection of β1−/− mice, only ∼2% of 2W1S-specific CD4 T cells were found in the BM relative to the spleen, whereas there was a 15-fold enhancement in Ag-specific CD4 T cells in the PP (∼1.5%). Thus, the high level of α4β7 aberrantly expressed by β1−/− CD4 T cells after activation is associated with decreased steady-state numbers of early memory CD4 T cells in the BM and increased numbers in intestinal sites.

Discussion

T cells express two distinct integrin heterodimers that contain the α4 integrin subunit, α4β7 and α4β1. It is critical to define the mechanisms that control the expression of α4β7 and α4β1 on T cells, as these integrins promote homing to the gut (α4β7) and to extraintestinal sites, such as the BM and brain (α4β1) (3, 17). In this study, we demonstrate that changes in the expression of β1 integrin reciprocally alter α4β7 expression on CD4 T cells. We show that this regulation occurs at the protein level, where the α4 integrin subunit preferentially pairs with the β1 integrin subunit when both β1 and β7 integrin are present. We identify the abundance of α4 integrin as the major driver of α4β7 expression on CD4 T cells and demonstrate its importance in RA-induced α4β7 upregulation. Finally, by tracking an endogenous Ag-specific population of CD4 T cells after infection, we demonstrate that the absence of β1 integrin results in enhanced α4β7 expression and altered localization of early memory CD4 T cells.

Naïve CD4 T cells express low levels of both the α4β1 and α4β7 integrins. When we crossed mice with floxed alleles of the β1 integrin gene with CD4-Cre transgenic mice, the loss of β1 integrin on naïve CD4 T cells was associated with increased expression of α4β7. These results are similar to recent findings from an independently derived line of conditional β1-deficient mice (10). We show that this change in α4β7 expression has functional significance, as β1−/− T cells exhibit enhanced adhesion to MAdCAM-1 in vitro and increased localization to PP in vivo. Because successful cell surface integrin expression requires intracellular α/β heterodimer formation, we suggest that the availability of the α4 integrin subunit for pairing regulates α4β7 integrin expression on T cells. Several lines of evidence suggest that the α4 integrin preferentially pairs with β1 integrin. First, we do not observe increased β1 integrin expression or function on β7-deficient CD4 T cells. Instead, loss of β7 integrin expression results in decreased cell surface expression of α4 integrin. This suggests that all available β1 integrin associates with α4 integrin, even when β7 integrin is present. Staining of permeabilized cells further supports this hypothesis, as there are abundant levels of intracellular β7 integrin, but not β1 integrin, in naïve CD4 T cells. The inability of β7 integrin overexpression to reduce β1 integrin expression in another system is also consistent with this model (4). Second, overexpression of β1 integrin on naïve CD4 T cells results in a dose-dependent decrease in α4β7 integrin expression without altering α4 integrin expression. Thus, increased levels of β1 integrin can effectively outcompete β7 integrin for association with the limiting amount of the α4 subunit expressed in CD4 T cells. Thus, there is a hierarchy of β subunit pairing to the α4 integrin, with a “dominant” β1 integrin subunit that modulates the expression of the other heterodimer, α4β7.

The functional relevance of this integrin subunit pairing hierarchy is revealed by our analysis of β1 and α4β7 integrin expression after CD4 T cell activation. Activation of control T cells with anti-CD3/CD28 Abs for 3 d resulted in increased β1 integrin expression and loss of α4β7 integrin expression even before the first cell division. These results are consistent with our overexpression data demonstrating β1 integrin as the dominant α4 integrin pairing partner. In contrast, β1−/− T cells exhibit dramatically elevated levels of α4β7 expression. Expression of α4 integrin on T cells is also increased after activation, although the level of surface α4 integrin was slightly lower on β1−/− T cells. This is likely due to the overall lower levels of β subunits available for pairing with α4 integrin in β1−/− T cells. These findings suggest that the preferential pairing of β1 integrin with α4 integrin is critical for the suppression of α4β7 expression after T cell activation.

Our work supports a model where β1 integrin expression modulates α4β7 expression by controlling the abundance of α4 integrin available to pair with β7 integrin. In a naïve T cell, all available β1 integrin pairs with α4 integrin and is expressed on the cell surface as α4β1. Any remaining free α4 integrin is then available for pairing with β7 integrin, resulting in a low level of α4β7 integrin expression on the cell surface. The identification of an intracellular pool of β7 integrin in naïve CD4 T cells suggests that β7 integrin is expressed in excess of the available α4 integrin. We also demonstrate that α4β7 cell surface expression is enhanced when the α4 integrin subunit is overexpressed in naïve T cells, even though mRNA levels for β7 integrin are not altered. This suggests that intracellular β7 integrin serves as a reservoir of β7 integrin available for pairing with free α4 integrin. Our model is consistent with microarray data that shows human, β7-high memory CD4 T cells have an increase in mRNA transcript for α4 integrin but not β7 integrin (40). The relative abundance of β1 integrin to α4 integrin is critical, as overexpression of both α4 and β1 integrin results in suppression, rather than induction, of α4β7 expression. In this situation, the excess β1 integrin subunits likely associate with the free α4 integrin subunits, thereby suppressing α4β7 heterodimer formation. Overall, our model predicts that the precise ratio of β1 to α4 subunit is critical for controlling the expression of α4β7. A ratio favoring β1 integrin results in suppression of α4β7, whereas a ratio favoring α4 integrin results in increased α4β7 expression.

Previous work has shown that RA produced by intestinal DCs induces α4β7 expression on T cells (21, 22), but the exact cellular mechanism for how this occurs is unknown. CD8 T cells activated by intestinal DCs are reported to have increased mRNA transcript for α4 integrin but not β1 or β7 integrin (41). We find that RA-treated, activated CD4 T cells have a similar pattern of enhanced α4 integrin protein and mRNA without alterations in β1 or β7 integrin mRNA. Thus, RA-induced α4β7 expression is driven by increased abundance of α4 integrin. As a result, the β1 to α4 subunit ratio favors the α4 integrin. Under these conditions, α4 integrin is no longer a limiting pairing partner. Thus, β7 integrin can pair with excess α4 integrin not bound to β1, resulting in increased α4β7 cell surface expression. Our finding that overexpression of α4 integrin mimics the effects of RA treatment on α4β7 expression supports this model. The precise balance between the β1 and α4 subunits remains critical, as the overexpression of β1 integrin in RA-treated CD4 T cells suppresses the induction of α4β7. In this study, the addition of excess β1 integrin elevates the β1 to α4 subunit ratio, resulting in
suppression of α4β7 expression. These results highlight the importance of the stoichiometry of the α4, β1, and β7 integrin subunits in determining the relative levels of α4β1 and α4β7 that are expressed on the surface of a T cell. This mechanism of regulation of integrin expression may also be applicable to other T cell subsets that express integrin subunits that share a common integrin subunit binding partner (42–45).

To examine changes in integrin expression after T cell activation in vivo, we used peptide-MHC class II tetramer-based enrichment approaches to monitor changes in integrin expression on a polymicrobial population of Ag-specific CD4 T cells after L. monocytogenes infection (36). This approach avoids possible alterations in activation kinetics, homing molecule expression, and memory generation and maintenance that have been reported when using high cell number adoptive transfer (46–49). In the spleen, we unexpectedly detected high levels of α4β7 on ∼50% of activated control CD4 T cells at the peak of the response (day 5). Although splenic DCs have been reported to induce α4β7 on CD8 T cells in vitro, this has not been reported for CD4 T cells in vivo (50). A transient increase in the availability of the α4 subunit after T cell activation in the spleen may explain the high percentage of β1 integrin expressing α4β7-high CD4 T cells recovered 5 d after infection. By day 18, the majority of activated control CD4 T cells expressed high levels of β1 integrin and low levels of α4β7, whereas a smaller subpopulation had a β1-low α4β7-high gut-homing phenotype (8–11%). Our results suggest that CD4 T cells with this gut-homing phenotype have a mechanism to suppress β1 integrin abundance at the protein and/or mRNA level. In contrast with this gut-homing phenotype have a mechanism to suppress whereas a smaller subpopulation had a expression of α4β7 on a subset of human CD4+ memory T cells with hallmarks of gut-trophism. J. Immunol. 151:717–729.


