Genetic perturbation of the putative cytoplasmic membrane-proximal salt bridge aberrantly activates α4 integrins

Yoichi Imai,1,2 Eun Jeong Park,1,2 Dan Peer,1,2 António Peixoto,2,3 Guiying Cheng,2,3 Ulrich H. von Andrian,2,3 Christopher V. Carman,4 and Motomu Shimaoka1,2

1Department of Anesthesia, Harvard Medical School, Boston, MA; 2Immune Disease Institute, Boston, MA; 3Department of Pathology, Harvard Medical School, Boston, MA; and 4Beth Israel Deaconess Medical Center and Department of Medicine, Harvard Medical School, Boston, MA

α4 integrins play a pivotal role in leukocyte migration and tissue-specific homing. The ability of integrins to bind ligand is dynamically regulated by activation-dependent conformational changes triggered in the cytoplasmic domain. An NMR solution structure defined a putative membrane-proximal salt bridge between the αβ3 integrin cytoplasmic tails, which restrains integrins in their low-affinity state. However, the physiological importance of this salt bridge in α4 integrin regulation remains to be elucidated. To address this question, we disrupted the salt bridge in murine germ line by mutating the conserved cytoplasmic arginine R\textsuperscript{GFFKR} in α4 integrins. In lymphocytes from knock-in mice (α\textsubscript{c}-R/A\textsuperscript{GFFKR}), α\textsubscript{4}\β\textsubscript{1} and α\textsubscript{4}\β\textsubscript{2} integrins exhibited constitutively up-regulated ligand binding. However, transmigration of these cells across VCAM-1 and MadCAM-1 substrates, or across endothelial monolayers, was reduced. Perturbed detachment of the tail appeared to cause the reduced cell migration of α\textsubscript{c}-R/A\textsuperscript{GFFKR} lymphocytes. In vivo, α\textsubscript{4}-R/A\textsuperscript{GFFKR} cells exhibited increased firm adhesion to Peyer patch venules but reduced homing to the gut. Our results demonstrate that the membrane-proximal salt bridge plays a critical role in supporting proper α4 integrin adhesive dynamics. Loss of this interaction destabilizes the nonadhesive conformation, and thereby perturbs the properly balanced cycles of adhesion and deadhesion required for efficient cell migration. (Blood. 2008;112:5007-5015)

Introduction

Integrins, αβ heterodimeric glycoproteins, constitute the largest family of cell-surface adhesion molecules that mediate diverse cell-cell and cell-matrix interactions, supporting a wide range of physiologic processes including development, immune regulation, hemostasis, and wound healing.1,2 A hallmark of the integrin family of adhesion receptors is their unique ability to dynamically up- and down-regulate their adhesiveness and, thereby, to coordinate the orchestrated cellular movements observed in leukocyte migration.3 This ability is achieved largely through conformational changes that are coupled to ligand binding.5 Intracellular signals, elicited by other receptors that sense external stimuli, trigger conformational changes in the cytoplasmic domain, which are then propagated across the plasma membrane to the extracellular domain.1,2 Under resting conditions, integrins exist largely in a low-affinity, nonadhesive conformation, and upon stimulation are converted to high-affinity conformations. The integrin cytoplasmic domain plays a pivotal role in modulating the activity of the integrins to bind ligand.4,5 The cytoplasmic domains of the α- and β-subunits in integrins are thought to associate with each other at the membrane-proximal regions in latent low-affinity state (Figure 1A left). This αβ cytoplasmic association functions as a “clasp” that restrains integrins in a default low-affinity conformation. Upon activation, binding to the integrin cytoplasmic domains of signaling molecules (eg, talin) triggers “unclasing,” which leads to the conversion from low-affinity to high-affinity conformations (Figure 1A right).6

Structural investigations using NMR revealed critical interactions at the association interface between αβ cytoplasmic domains.7 The arginine residue in the conserved GFFKR sequence (termed R\textsuperscript{GFFKR} in this study, Figure 1B) at the membrane-proximal region of the α-subunit forms a putative salt bridge with the β-subunit. This putative membrane-proximal salt bridge is thought to constitute a critical interaction that contributes to clasping the αβ cytoplasmic domains and thereby restrains integrin activation (Figure 1A left). R/A\textsuperscript{GFFKR}, the alanine substitution of R\textsuperscript{GFFKR} that would disrupt the clasping salt bridge selectively, constitutively activated α\textsubscript{4}\β\textsubscript{1} and α\textsubscript{4}\β\textsubscript{2} in transfectants.6 Furthermore, these in vitro results using transfectants have been supported by findings in knock-in mice for α\textsubscript{4}\β\textsubscript{2} and in patients for α\textsubscript{4}\β\textsubscript{3}. The deletion of the α\textsubscript{4}-GFFKR sequence in germ line persistently activated α\textsubscript{4}\β\textsubscript{2} in a strain of knock-in mice (L\textsubscript{fa}-1\textsuperscript{D723A}).9 β\textsubscript{2}, D723H, the mutation found in a group of patients with inherited thrombocytopenia, increased activity of α\textsubscript{4}\β\textsubscript{3} integrin in patients’ platelets.10

In contrast to α\textsubscript{4}\β\textsubscript{3} integrins, the physiological role of the putative membrane-proximal salt bridge remains to be elucidated in α\textsubscript{4} integrins (α\textsubscript{4}\β\textsubscript{1} and α\textsubscript{4}\β\textsubscript{2}). α\textsubscript{4} integrins play a pivotal role in lymphocyte adhesion to, and transmigration across, the endothelium during physiologic lymphocyte migration, including
membrane-proximal cytoplasmic domains of selected integrins are shown with 2 parallel dashed lines. (B) Amino acid sequence alignment of the (RGFFKR) are shown in bold. (C) Targeted insertion of the floxed ACN cassette using sperm-specific ACE promoter, 25 mutated exon 28 (28\textsuperscript{+}), and UTR into the -subunit, converting the extracellular domains to the active conformation (right). The cytoplasmic proteins such as talin (not shown), triggers unclasping of the cytoplasmic conformation (left). Disruption of the salt bridge, which is induced via binding of cytoplasmic domains, maintaining the extracellular domains in a default inactive state, and rapidly up-regulated only upon activation with exogenous stimuli.\textsuperscript{13,14} Thus, it is of great importance to investigate the physiological significance of the putative membrane-proximal salt bridge in regulating \(\alpha_4\) integrin activation.

Here we attempt to disrupt the putative membrane-proximal salt bridge in the integrin \(\alpha_4\) subunit in mouse germ line. The deletion of the entire GFFKR sequence, which was previously used in Lf41\textsuperscript{40} knock-in mice,\textsuperscript{9} would not only disrupt the salt bridge via R\textsuperscript{GFFKR}, but also substantially perturb the \(\alpha/\beta\) cytoplasmic association interface. Thus, to selectively disrupt the putative membrane-proximal salt bridge, we knocked in a point mutation \(\alpha_4\text{-}RA/GFFKR\). We have shown that compared with wild type (WT), lymphocytes from \(\alpha_4\text{-}RA/GFFKR\) mice exhibit constitutively enhanced cell adhesion to VCAM-1 and MAdCAM-1, thus demonstrating the importance of the membrane-proximal salt bridge in restraining integrin activation in \(\alpha_4\beta_1\) and \(\alpha_4\beta_7\). Furthermore, \(\alpha_4\text{-}RA/GFFKR\) lymphocytes exhibit perturbed lateral migration on, and transmigration across, VCAM-1 and MAdCAM-1 substrates as well as delayed transendothelial migration. In vivo, \(\alpha_4\text{-}RA/GFFKR\) lymphocytes display an increased capacity to firmly adhere to Peyer patch (PP) venules but a reduced capacity to home to the GALT. These data support the idea that the putative cytoplasmic membrane-proximal bridge plays a pivotal role in the balanced regulation of \(\alpha_4\) integrin adhesiveness to facilitate cell migration.

**Methods**

Supplemental methods are available in Document S1 (available on the Blood website; see the Supplemental Materials link at the top of the online article).

All experiments using mice were approved by the Institutional Review Board of the Immune Disease Institute.

**Flow chamber assays**

Flow chamber assays were performed as previously described\textsuperscript{15} using mouse VCAM-1-Fc (10 \mu g/mL), MAdCAM-1-Fc (10 \mu g/mL), or ICAM-1/Fc (50 \mu g/mL) immobilized on plastic. Cells were resuspended at 10^6/mL for interactions with VCAM-1 and ICAM-1, and at 4 x 10^5/mL for interactions with MAdCAM-1, in Hanks balanced salt solution, 10 mM HEPES, 0.5% BSA containing 1 mM Mg\textsuperscript{2+}/Ca\textsuperscript{2+} and 1 mM Mn\textsuperscript{2+}. Cells were infused into the chamber and allowed to accumulate on the substrate at a shear stress of 0.3 dyne/cm\textsuperscript{2} for 45 seconds. Wall shear stress was increased incrementally every 10 seconds. Microscopic images of cells under flow were recorded and analyzed off-line as previously described.\textsuperscript{16}

**Transwell assays**

Transmigration was studied as described previously with minor modifications using a modified Boyden chamber assay with a 6.5-mm Transwell tissue culture system (Corning, Corning, NY) containing a permeable support insert with a 5-\mu m pore size.\textsuperscript{17} In brief, Transwell inserts were coated with 40 \mu g/mL murine MAdCAM-1-Fc, VCAM-1-Fc, or ICAM-1-Fc. Splenocytes (10^6) were added to the upper chamber and allowed to transmigrate for 4 hours to the lower chamber that contained 2 \mu g/mL CXCL12. Transmigrated cells were counted using a FACScan flow cytometer (BD Biosciences, San Jose, CA).

**Transendothelial migration under shear stress**

Transendothelial migration (TEM) under shear stress was studied as previously described\textsuperscript{18} using bEnd.3 mouse brain endothelioma cells (passages 6 to 10; ATCC, Manassas, VA). bEnd.3 cells were plated at 0.5 x 10^6 cells per 40 mm on circular coverslips coated with 20 \mu g/mL fibronectin and cultured for 48 hours. The cells were stimulated for 24 hours with 50 ng/mL TNF-\alpha, and 2 \mu g/mL CCL21 was overlaid for 15 minutes.

**Figure 1.** A model for integrin activation regulated by the membrane-proximal cytoplasmic salt bridge and generation of \(\alpha_4\text{-}RA/GFFKR\) mice in which the cytoplasmic salt bridge is disrupted. (A) A positively charged RGFFKR (a circled plus) forms the membrane-proximal salt bridge with a negatively charged residue(s) (a circled minus) in the \(\beta\)-subunit. The salt bridge facilitates clasping of the \(\alpha/\beta\) cytoplasmic domains, maintaining the extracellular domains in a default inactive conformation (left). Disruption of the salt bridge, which is induced via binding of cytoplasmic proteins such as talin (not shown), triggers unclasping of the cytoplasmic domains, converting the extracellular domains to the active conformation (right). The \(\alpha\) and \(\beta\)-subunits are labeled in the extracellular domains. The plasma membrane is shown with 2 parallel dashed lines. (B) Amino acid sequence alignment of the membrane-proximal cytoplasmic domains of selected integrin \(\alpha\)-subunits. A conserved GFFKR motif is highlighted. The arginine residues in the GFFKR motif (R\textsuperscript{GFFKR}) are shown in bold. (C) Targeted insertion of the floxed ACN cassette containing both the flox\textsuperscript{g} gene and the Cre-recombinase gene under the control of the sperm-specific ACE promoter,\textsuperscript{25} mutated exon 28 (28\textsuperscript{+}), and UTR into the Itga4 locus, followed by deletion of the ACN cassette. The configurations of the targeting vector, \(\alpha_4\) integrin genomic locus, the targeted allele following homologous recombination, and the ACN-deleted allele are shown. Exons are displayed as black boxes and the floxed ACN cassette, UTR, and diphtheria toxin cassette (DT) are displayed as white boxes. loxP sites are displayed as triangles. The external probe containing exon 24, which was used to verify the targeting event, is indicated. The map displays the following restriction sites: E indicates EcoRI; S, SacI; Sa, Sacd; X, XbaI; and X*, engineered XbaI. (D) Genotyping and confirmation of the deleted ACN cassette by PCR. PCR bands are shown for wild-type (WT/WT, 440 bp), heterozygous (Ko/WT, 550 and 440 bp), and homozygous (Ko/Ko, 550 bp) samples.
on the bEnd.3 cell monolayer. Coverslips with endothelium were then assembled into an FCS2 parallel wall laminar shear flow chamber (Biotechs, Butler, PA). For transendothelial migration analysis, splenocytes were perfused for 2 minutes over the monolayer at 0.25 dyne/cm² to allow accumulation. The flow rate was then increased to 2 dyne/cm² and kept constant for an additional 15-minute period during which time the images were recorded. Cell migration was visualized using a 10× phase-contrast objective and images were recorded using a CCD camera at a rate of 1 frame per 5 seconds. Cell adhesion and transmigration were assessed in the resulting videos by counting the number of lymphocytes adherent and laterally migratory over the apical surface of the endothelium (bright diffraction in phase-contrast imaging), as well as those that underwent transmigration to the subendothelial space (transition to a dark appearance in phase-contrast imaging).

**Live-cell imaging**

Live-cell imaging of laterally migrating cells on VCAM-1 and MAdCAM-1 substrates was performed as previously described. Briefly, 20 μg/mL VCAM-1-Fc or MAdCAM-1-Fc with 16 μg/mL CXCL12 was coated on Delta T chambers (Biotechs) at 4°C overnight. IL-15–treated memory/effector T cells were generated as previously described. After washing with L-15 medium (Cambrex Bioscience, East Rutherford, NJ), cells were added to the chambers and differential interference contrast (DIC) images were acquired using an Axiovert S200 epifluorescence microscope (Carl Zeiss, Heidelberg, Germany) equipped with a 63× oil objective coupled to an Orca CCD camera (Hamamatsu, Okyama City, Japan) at a frame rate of 15 seconds per frame. Cell migration was analyzed as previously described.

**Confocal microscopy**

Confocal microscopy was performed as previously described. Briefly, cells spread on 20 μg/mL VCAM-1 or MAdCAM-1 were cocultivated with 10 μg/mL CXCL12 and fixed and stained with mAb to α4 integrin (PS/2) followed by Cy3-conjugated goat anti–rat IgG and phalloidin–Alexa 488 for actin. Confocal imaging was conducted on a Radiance 2000 Laser-scanning confocal system (Bio-Rad Laboratories, Hercules, CA) using a microscope (model BX50/WH; Olympus, Melville, NY) with a 100× water-immersion objective. Imaging processing was performed with OpenLab software (Improvision, Waltham, MA). Multiple randomly selected fields were imaged for each condition by collecting serial sections through the entire sample Z-axis. These were then projected as complete stacks. For morphologic analysis, cell polarity was identified as the actin-rich crescent-shaped leading edge. The opposing trailing edge was assessed for the presence of extended (> 1 μm) tethers that were rich in both actin and α4-integrin staining. The number of such projections was counted for each of at least 20 cells in randomly selected fields and averaged.

**Epifluorescence intravital microscopy**

Intravital microscopy of PPs was performed as previously described. Briefly, C57BL/6J mice were anesthetized and a bowel segment of the small intestine was positioned for epifluorescence intravital microscopy, to record inflammation. To induce gut inflammation, mice were fed for 6 days with 5% (wt/vol) DSS in drinking water. On day 5, the same numbers of donor splenocytes (2 × 10⁷) from WT and KI mice labeled with different dyes (CFSE and CMTMR) were mixed and injected intravenously. Eighteen hours later, mice were killed and all lymphoid and nonlymphoid organs were harvested and analyzed as described. In some experiments, fluorescent dyes were switched.

**Statistical analysis**

Data are expressed as the mean plus or minus SEM for each group. The Student t test was used for statistical analyses unless otherwise indicated.

**Results**

**Generation of knockin mice in which the membrane-proximal salt bridge is disrupted at the α4 cytoplasmic domain**

To disrupt the critical salt bridge that stabilizes the αβ cytoplasmic association in α4 integrins in vivo, we used a replacement-type gene-targeting strategy to mutate R⁴⁴⁴K⁴⁴⁵ to alanine in the integrin α4 gene (Itga4), thereby generating an Itga4-R⁴⁴⁴K⁴⁴⁵ mutated allele in embryonic stem (ES) cells (Figure 1C). We obtained the correct integration of the Itga4-R⁴⁴⁴K⁴⁴⁵ allele into the mouse germ line (Figure 1D and data not shown) and designated mice homozygous for the mutant α4 allele as α4-R⁴⁴⁴K⁴⁴⁵K⁴⁴⁵. These mice were born under normal Mendelian ratios, were fertile, and did not exhibit gross abnormalities.

**Increased ligand binding by α4β1 and α4β7 in α4-R⁴⁴⁴K⁴⁴⁵K⁴⁴⁵ lymphocytes**

Lymphocytes from the spleen of α4-R⁴⁴⁴K⁴⁴⁵K⁴⁴⁵ mice showed approximately 50% reduction of α4 and α4β1 expression on the cell surface, compared with WT cells (Figure 2A). Only a slight decrease in β1-subunit expression was observed. Expression of the α4β2 integrin, L-selectin, and the activation markers CD69 and CD25 was not affected (Figure 2A and data not shown). Similar results were seen in lymphocytes from peripheral lymph nodes (PLNs) and Peyer patches (PPs) (Figure S1).

Despite the reduced cell-surface α4 integrin expression in α4-R⁴⁴⁴K⁴⁴⁵K⁴⁴⁵ lymphocytes, WT and α4-R⁴⁴⁴K⁴⁴⁵K⁴⁴⁵ cells showed comparable levels of total α4 integrin expression, which included cell-surface and intracellular expression, as determined by immunofluorescent cytometry analyses of permeabilized cells (Figure 2B). Quantitative reverse-transcription–polymerase chain reaction (RT-PCR) showed that mRNA expression levels of the α4 subunit in WT and α4-R⁴⁴⁴K⁴⁴⁵K⁴⁴⁵ splenocytes were comparable (Figure 2C). Similar results with α4β2 (ie, reduced cell-surface and comparable total expression) were also obtained in Lfu-1 knockout mice, in which the α4 GFFKR sequence had been deleted. As previously shown in the α4-β2 subunit, heterodimer formation and subsequent translocation to the cell surface might be partially perturbed in the context of the mutant α4 subunit, possibly through effects on integrin conformation.

To investigate the possibility that a potential increase in an intracellular pool of mutant α4 integrin subunit might activate the unfolded protein response (UPR) that could modify cellular metabolism and functions, we studied UPR in WT and α4-R⁴⁴⁴K⁴⁴⁵K⁴⁴⁵ splenocytes. Using quantitative RT-PCR, we measured the
mRNA levels of GRP78 and the spliced form of XBP-1, both of which are known to be up-regulated in response to UPR. We have shown that WT and \( H_{9251} \quad H_{4-R/AGFFKR} \) cells expressed comparable levels of GRP78 and the spliced XBP-1 (Figure 2D). Thus, at least in this experimental setting, we did not find evidence that \( H_{9251} \quad H_{4-R/AGFFKR} \) cells are subjected to an increased UPR, compared with WT cells.

We investigated the adhesive interactions of \( H_{9251} \quad H_{4 integrins} \) with their ligands VCAM-1 and MAdCAM-1 under physiological shear conditions using a parallel wall flow chamber. A fraction of WT splenic lymphocytes firmly adhered to VCAM-1 and MAdCAM-1 substrates in approximately 1 mM Ca\(^{2+}\)/Mg\(^{2+}\), the cation condition in which integrins are predominantly maintained in a nonadhesive state (Figure 3A,B). Under the same setting, approximately 3 times as many \( H_{9251} \quad H_{4-R/AGFFKR} \) lymphocytes firmly adhered as did WT cells, demonstrating increased basal adhesiveness by \( H_{9251} \quad H_{4 integrins} \) in \( H_{9251} \quad H_{4-R/AGFFKR} \) lymphocytes (Figure 3A,B). In the current experimental setting, we observed only a small fraction of WT and \( H_{9251} \quad H_{4-R/AGFFKR} \) lymphocytes that rolled on VCAM-1 and MAdCAM-1 substrates (Figure S2). Increased adhesiveness was specific to the \( H_{9251} \quad H_{4 integrins} \), as \( H_{9251} \quad L_{2} \) in \( H_{9251} \quad H_{4-R/AGFFKR} \) remained as latent in binding to ICAM-1 as did WT cells (Figure S3). To study the relative contributions of \( H_{9251} \quad H_{4 and \( H_{9251} \quad H_{4 integrins} \) to the enhanced adhesion by \( H_{9251} \quad H_{4-R/AGFFKR} \) lymphocytes, we used a panel of function-blocking mAbs. Adhesion of \( H_{9251} \quad H_{4-R/AGFFKR} \) and WT cells to VCAM-1 was blocked completely with anti-\( H_{9251} \quad H_{4 mAb} \), and partially with either anti-\( H_{9251} \quad H_{4 or anti-\( H_{9251} \quad H_{4-R/AGFFKR} \) blocking mAb, confirming that the interaction was mediated by both \( H_{9251} \quad H_{4 and \( H_{9251} \quad H_{4 integrins} \) in the presence of either anti-\( H_{9251} \) or
anti-α4β7 blocking mAb, adhesion of α5-RA/GFFKR cells was greater than WT, demonstrating that the adhesiveness of both α4β7 and α5β1 integrins to VCAM-1 had increased (Figure 3E,G,I). By contrast, adhesion of α5-RA/GFFKR and WT cells to MadCAM-1 was blocked completely with anti-α5 mAb, or by anti-α5β1 blocking mAb, indicating that enhanced adhesion of α5-RA/GFFKR lymphocytes to MadCAM-1 was solely mediated by the α5β1 integrin (Figure 3F,H,J). Upon stimulation with Mn2+ capable of mimicking inside-out signaling to induce high-affinity integrin conformations, WT cells exhibited increased firm adhesion to VCAM-1 and MadCAM-1 (Figure 3C,D). Mn2+ stimulation enhanced firm adhesion of α5-RA/GFFKR cells to VCAM-1 and MadCAM-1 (Figure 3C,D), suggesting that α5 integrins in α5-RA/GFFKR cells are not fully activated and are further activatable with agonists. These results convincingly demonstrated that the disruption of the α4 cytoplasmic membrane-proximal salt bridge increased the adhesiveness of both α4β1 and α5β1, despite the reduced cell-surface expression of mutant α4 integrins. Similar results were obtained with cells from PLNs and PPs (data not shown).

Disturbed transmigration of α5-RA/GFFKR lymphocytes through VCAM-1 and MadCAM-1

As an appropriate balance between adhesion and deadhesion is thought to be critical for efficiently coordinated cell migration, we studied how the persistently increased adhesion of α5-RA/GFFKR cells affected transmigration by using a Transwell assay.17 WT and α5-RA/GFFKR cells transmigrated equally well through an ICAM-1–coated Transwell insert into a chemokine CXCL12–containing lower chamber, demonstrating normal migration behavior in general and normal coordination of α5β1 integrin adhesiveness, specifically in α5-RA/GFFKR (Figure 4A). In contrast, when Transwell inserts were coated with VCAM-1 or MadCAM-1, α5-RA/GFFKR cells showed approximately 50% reduction of transmigrated cells compared with WT (Figure 4B,C).

Disturbed transendothelial migration of α5-RA/GFFKR lymphocytes under shear stress

Transendothelial migration (TEM) is a critical step that regulates lymphocyte entry into lymph nodes as well as leukocyte accumulation into inflamed tissues. During lymphocyte TEM, α5 integrins cooperate with α6β1 integrin and other adhesion molecules.29 The presence of shear stress enhances the capacity of lymphocytes to transmigrate through endothelial monolayers.30 To study transendothelial migration by WT and α5-RA/GFFKR lymphocytes under physiologic shear stress, we used monolayers of bEnd.3 mouse endothelial cells assembled into a flow chamber. The monolayers of bEnd.3 cells were treated with TNF-α to up-regulate surface expression of integrin ligands such as VCAM-1, MadCAM-1, and ICAM-1. A CCL21 chemokine was immobilized on the endothelial surface to facilitate adhesion to, and transmigration through, endothelial cells. Identical numbers of WT and α5-RA/GFFKR cells were infused into the chamber and allowed to accumulate on endothelial monolayers for 2 minutes under 0.25 dyne/cm2 shear stress. The migratory behaviors of adherent lymphocytes were then recorded under 2.0 dyne/cm2 shear stress for 15 minutes. Although α5-RA/GFFKR cells were more adhesive to VCAM-1 or MadCAM-1 in the absence of chemokine stimulation (Figure 3A,B), comparable numbers of WT and α5-RA/GFFKR cells accumulated on bEnd.3 endothelial monolayers (WT, 89.7 ± 4.0; KI, 94.3 ± 2.5 cells per field). Surface-displayed CCL21 is likely to activate α4β7 and α5 integrins on lymphocytes to support firm adhesion on bEnd.3 cells that simultaneously express VCAM-1, MadCAM-1, and ICAM-1. However, when allowed to transmigrate at 2.0 dyne/cm2 shear stress for 15 minutes, approximately 50% as few α5-RA/GFFKR cells underwent TEM as did WT cells (Figure 4D).

Perturbed lateral migration of α5-RA/GFFKR lymphocytes on VCAM-1 and MadCAM-1 substrates

To investigate the mechanism(s) by which TEM of α5-RA/GFFKR lymphocytes was perturbed, we studied lateral migration with live-cell time-lapse video microscopy. Although we tried to use naive lymphocytes in which basal integrin adhesiveness is maintained at low levels, they failed to show an efficient lateral migration on integrin ligands in response to chemokine. Similar observations were made in previous reports.31 As an alternative, we used IL-15–treated memory effector T cells, in which integrins are known to show little basal ligand binding, but readily up-regulate upon chemokine stimulation.32

WT T cells migrated smoothly on VCAM-1/CXCL12 substrates (Movie S1). By contrast, α5-RA/GFFKR T cells migrated poorly on VCAM-1/CXCL12 substrates (Video S2). Whereas WT cells migrating on VCAM-1 were polarized normally, displaying a typically hand mirror–like shape with a flattened leading edge followed by a short tail, α5-RA/GFFKR T cells migrating on VCAM-1 exhibited extremely stretched tails enriched in α5 integrin (Figure 5A). Close image analysis reveals (Figure 5B) that the most remarkable morphologic distinction between 2 types of cells is the strong presence of α5 integrin–rich trailing edge tethers (Figure 5B arrows) in α5-RA/GFFKR cells but not WT cells. Quantitative image analysis demonstrated that α5-RA/GFFKR cells displayed significantly more tethers than WT cells (Figure 5C). Similar results were obtained in T cells migrating on MadCAM-1 substrates (Figure 5A,C). These results strongly suggest that the perturbed detachment of the tail accounts for decreased TEM of α5-RA/GFFKR cells across bEnd.3 endothelial monolayers.
Increased firm adhesion of α4R/AGFFKR lymphocytes to PP venules in vivo

We have demonstrated proof of principle that the aberrantly activated α4R/AGFFKR perturbs TEM across bEnd.3 cell line under shear stress; however, TNF stimulation of, and apical addition of CCL21 to, bEnd.3 cells might not fully recapitulate in vivo phenotypes of HEVs in the gut during normal lymphocyte homing. To study the in vivo adhesive interactions of α4R/AGFFKR lymphocytes with HEVs in the GALT, such as PPs, we performed an intravital microscopic investigation of PP venules.21

We injected calcein-labeled α4R/AGFFKR or WT splenocytes into anesthetized WT syngeneic recipient mice that had been prepared for intravital microscopy of PPs. The injected cells were then observed and video recorded entering and interacting with PP venules. Adhesive interactions of α4R/AGFFKR or WT donor splenocytes with PP venules were analyzed off-line. We found that α4R/AGFFKR and WT cells rolled on PP venules at similar frequencies (Figure 6A) and comparable velocities (Figure 6C). However, α4R/AGFFKR cells exhibited higher capacity to firmly adhere to PP venules than WT cells (Figure 6B). These data demonstrated that despite reduced cell-surface expression of α4 integrin, α4R/AGFFKR cells showed increased firm adhesion to PP venules in vivo.

Suppression of α4R/AGFFKR lymphocyte migration to the gut

α4 integrin plays a pivotal role in a physiologic lymphocyte recirculation through the GALT as well as a pathologic lymphocyte accumulation to inflamed gut.11,12 To study the impact of aberrantly activated α4 integrins on lymphocyte migration to the gut, we used a competitive homing assay. We began with investigating lymphocyte homing to noninflamed tissues. Splenocytes from WT and α4R/AGFFKR mice were fluorescently labeled with CFSE and CMTMR, respectively. Equal numbers of WT and α4R/AGFFKR cells were mixed and intravenously administered into C57BL/6J-CD45.1+ mice. Eighteen hours later, WT...
and α4-R/AGFFKR lymphocytes homed equally well to SP and PLNs (Figure 7A), and comparable fractions of WT and α4-R/AGFFKR lymphocytes were present in peripheral blood (WT, 0.59% ± 0.18%; and α4-R/AGFFKR, 0.57% ± 0.18%; of injected cells [mean ± SEM, n = 6]). By contrast, homing of α4-R/AGFFKR lymphocytes to the gut (including MLNs, PPs, small and large intestines) was reduced by approximately 50% compared with WT (Figure 7A). To study whether a subpopulation of α4-R/AGFFKR cells with regard to α4 surface expression might selectively be blocked to enter tissues, we compared α4 integrin expression of WT and α4-R/AGFFKR cells in donor cells before injection and those homed to tissues. The ratio of α4 expression in WT and KI cells is comparable in both donor cell populations (Table S1).

We then investigated lymphocyte homing to inflamed gut. We induced mild colitis in C57BL/6J-CD45.1+ recipient mice by feeding them for 6 days with 3% DSS. At day 5, mice exhibited approximately 4% body weight loss and intestinal edema formation (not shown). We performed a new set of in vivo competitive homing assays using the recipient mice with DSS-induced colitis. Eighteen hours after injection of equal numbers of fluorescently labeled WT and α4-R/AGFFKR cells, cells were harvested from inflamed gut and other organs, and the homing indices were then determined. WT and α4-R/AGFFKR lymphocytes homed equally well to SP and PLNs. However, α4-R/AGFFKR lymphocytes exhibited approximately 50% reduced homing to the gut, compared with WT (Figure 7B). These results demonstrated that the aberrant activation of α4 integrins perturbed lymphocyte homing to both uninflamed and inflamed gut.

**α4-R/AGFFKR mice contain reduced gut lymphocytes**

The capacity of lymphocytes to migrate/home represents an important factor in regulating the size and cellularity of lymphoid tissues, which could be affected by the perturbed migration of lymphocytes in α4-R/AGFFKR mice. Therefore, we studied the distribution of lymphocytes in lymphoid organs. Macroscopic examination showed that the PPs from α4-R/AGFFKR mice were smaller than those of WT (WT, 1.4 ± 0.1 mm; α4-R/AGFFKR, 0.9 ± 0.1 mm; diameter, P < .01), whereas SP, PLNs, and MLNs were indistinguishable from one mouse strain to another (data not shown). Consistent with the results of our in vivo homing assay, α4-R/AGFFKR mice contained significantly fewer lymphocytes in the gut (ie, PP and IEL compartments) compared with WT (Table 1).

**Discussion**

We have demonstrated that disruption of the putative cytoplasmic membrane-proximal salt bridge leads to constitutively activated α4β1 and α4ββ integrins. Our results with α4-R/AGFFKR knock-in mice have convincingly demonstrated that the membrane-proximal salt bridge plays a critical role in restraining default low-affinity state of α4 integrins in physiologically relevant settings.

The importance of the putative membrane-proximal salt bridge in β1 integrins including α4β1 remains to be questioned. Czuchra et al previously knocked in an alanine substitution, in the β1 subunit, of a membrane-proximal aspartate D759 that would form a salt bridge with R⁴GFFKR in the α4-subunit. Despite the hypothesis that the mutation would activate β1 integrins, β1-D759A keratinocytes failed to show an increase in cell adhesion to β1 integrin ligands; and β1-D759A bone marrow cells did not show activation-dependent conformational changes in the β1-subunit. These results led Czuchra et al to argue that...
the membrane-proximal salt bridge might not be important for physiologic regulation of β1 integrins. It has yet to be directly demonstrated whether αβ1 adhesiveness in β1-D759A mice is enhanced; however, the effectiveness of perturbing the membrane-proximal salt bridge in αβ2 might substantially differ in β1-D759A and α4R/AGFFKR. The NMR solution structure for the α5β1 cytoplasmic domain showed that RAGFFKR (ie, α5R995) interacts not only with the membrane-proximal aspartate (β1-D723), but also with 2 other membrane-proximal conserved residues, histidine (β1-H722) and glutamate (β1-E726). Therefore, in β1-D759A mice, perturbation by the D759A mutation might be at least partially compensated by intact β1-H758 and/or β1-E762, thereby creating a much milder effect on the adhesiveness of β integrins. By contrast, the R/AGFFKR mutation disrupts all 3 electrostatic interactions, thereby inducing more robust activating phenotypes. Alternatively, R/AGFFKR mutation might destabilize the cytoplasmic αβ association via perturbing the local structural integrity of the α5 cytoplasmic domain. Future studies are necessary to determine the structure of αβ2 and αβ7 cytoplasmic domains.

Persistently increased adhesion of α4R/AGFFKR cells perturbed transmigration through VCAM-1 and MAdCAM-1. An appropriate balance between adhesion at the leading edge and deadhesion at the trailing edge is important for efficient cell migration. As shown by elongated tails of R/AGFFKR cells laterally migrating on VCAM-1 and MAdCAM-1, perturbed detachment at the trailing edge is likely to suppress directional forward movements of α4R/AGFFKR cells. Our results are consistent with previous studies involving either other strains of Lfa-1 

In summary, using knock-in mice we have demonstrated that the putative membrane-proximal cytoplasmic salt bridge is critical for maintaining default nonadhesive states of αβ1 and αβ7 integrins and consequently in supporting properly regulated integrin adhesive dynamics. The membrane-proximal salt bridge constitutes an important regulatory component within the integrin, one that facilitates the appropriate adhesion and deadhesion balance required for efficient and coordinated migration of lymphocytes to the gut.

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Authorship

Contribution: Y.I., E.J.P., and C.V.C. designed and performed research, analyzed data, and wrote the paper; D.P., A.P., and G.C. designed and performed research and analyzed data; U.H.A. designed research and analyzed data; and M.S. designed research, analyzed data, and wrote the paper.

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Correspondence: Motomu Shimaoka, 200 Longwood Avenue, Boston, MA 02115; e-mail: shimaoka@idi.harvard.edu.
Genetic perturbation of the putative cytoplasmic membrane-proximal salt bridge aberrantly activates $\alpha_4$ integrins

Yoichi Imai, Eun Jeong Park, Dan Peer, António Peixoto, Guiying Cheng, Ulrich H. von Andrian, Christopher V. Carman and Motomu Shimaoka

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