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α1β1 Integrin-Mediated Adhesion Inhibits Macrophage Exit from a Peripheral Inflammatory Lesion

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Integrins are adhesion molecules critical for the recruitment of leukocytes from blood into peripheral tissues. However, whether integrins are also involved in leukocyte exit from peripheral tissues via afferent lymphatics to the draining lymph node remains poorly understood. In this article, we show that adhesion by the collagen IV–binding integrin α1β1 unexpectedly inhibited macrophage exit from inflamed skin. We monitored macrophages exiting mouse footpads using a newly developed in situ pulse labeling technique. Blockade of α1β1 integrin or genetic deletion (Itga1−/−) increased macrophage exit efficiency. Chemotaxis assays through collagen IV showed more efficient migration of Itga1−/− macrophages relative to wild type. Given that macrophages are key orchestrators of inflammation, α1β1 integrin adhesion may represent a mechanism for regulating inflammatory responses by controlling macrophage exit or persistence in inflamed tissues.

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The family of integrins associated with the β1 subunit (CD29) includes several that bind ECM molecules, and some of these are found on leukocytes, including macrophages (7). This β1 subset of integrins was first identified on T cells but appeared only after days or weeks of in vitro culture; hence they were named very late Ags 1–6, and since that time, additional α subunits have been found that can associate with the β1 chain (7–10). Very late Ag 1 (α1β1 integrin or CD49a/CD29) on T cells was the first of the β1 group to be characterized, and it was found to bind ECM, in particular, collagen IV, but not collagen I (11). Later studies further characterized α1β1 integrin expression on T cells, as well as confirming its ability to adhere to collagen IV (12–14). The α1β1 integrin was also shown to be important in inflammatory responses, required for T cell–mediated inflammation including delayed-type hypersensitivity and resistance to viral infection (13, 15). More recently, data have emerged demonstrating a critical role for α1β1 integrin in macrophage activation showing that the binding of Semaphorin7A (Sema7A) expressed on T cells to α1β1 integrin expressed on macrophages is required for effective macrophage inflammatory cytokine secretion (16).

Current models hold that when leukocyte integrins bind their ligands, this facilitates cell migration. Therefore, integrins are generally viewed as adhesion molecules with a promigratory function. Whether integrin-mediated adhesion can also inhibit leukocyte migration is less clear. Intriguingly, data generated from integrin-deficient dendritic cells demonstrated normal migration through noninflamed extravascular tissues (6). This suggests the possibility that in the appropriate biological context, integrins might play an alternate adhesive role as “anchoring” molecules, inhibiting rather than facilitating leukocyte migration. We investigated the function of α1β1 integrin in an inflammatory model using a newly developed in situ pulse labeling technique. Our results show that the collagen IV–binding integrin α1β1 inhibits macrophage exit from a peripheral inflammatory lesion.

Materials and Methods

Animals

α1 integrin knockout mice (Itga1−/−) bred onto a C57BL/6 background for at least 12 generations were a gift from A. Pozzi. For experiments, C57BL/6 (Jackson Laboratories) and Itga1−/− mice were between 8 and 12 wk old. All animal experiments were approved by the University Health

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Abbreviations used in this article: ECM, extracellular matrix; EP, ethylisopropylidate; IFPL, in situ pulse labeling; LN, lymph node; Sema7A, Semaphorin7A; S1P, sphingosine-1-phosphate.
Network Animal Care Committee and performed according to institutional and federal guidelines.

**Footpad injections and in situ pulse labeling**

Con A (20 μg in 20 μl PBS; Sigma) was injected into mouse footpads using a 50-μl Hamilton syringe. The fluorescent dye CFSE (Invitrogen Molecular Probes) was prepared according to the manufacturer’s instructions and further diluted in PBS to obtain a final concentration of 1.44 mM. Mice were lightly anesthetized using isoflurane via nosecone, and a tourniquet was applied at the distal calf. CFSE (10 μl) was injected into a Con A–infilled footpad using a 10-μl Hamilton syringe fitted with a custom-made 33-gauge needle. The tourniquet was removed 15 min after the CFSE injection, and mice were returned to cages. In some experiments, inflamed footpads were first injected with Ab (10 μg Ha31/8 or control Ab, Armenian Hamster IgG2, λ1, both NA/LE; BD Pharmingen), Obbtustatin (10 μg; a gift from C. Marcinkiewicz), or pertussis toxin (List Biochemicals). After 3 min, a tourniquet was applied and in situ pulse labeling (ISPL) was performed.

**Cell isolation**

Single-cell suspensions were prepared from mouse footpads and popliteal LNs. In both cases and regardless of treatment, cells excluded propidium iodide and there was no difference between Itga1+/+ and Itga1−/− staining. For isolation of cells from the footpad, mice were euthanized by carbon dioxide or isoflurane overdose and perfused with ice-cold PBS via the left ventricle. Footpad tissue (~8 by 4 mm) was excised from the plantar surface, cut into small squares (~1 mm), and digested (1 h, 37°C) with Liberase CI (250 μg/ml; Roche) and DNase I (50 μg/ml; Roche) in DMEM (Sigma). EDTA (10 mM final concentration) was then added and samples were incubated on ice for 5 min. Digested footpad tissues were then gently dissociated using the plunger of a 1-ml syringe (BD) through a 200-μm pore wire mesh (custom made) and then further filtered through a 40-μm nylon mesh (Becton Dickinson (BD)). Popliteal LNs were isolated and gently dissociated using small forceps. LN tissue was digested (20 min, 37°C) with collagenase D (2 mg/ml) in RPMI medium (Wisent). Digested LNs were then incubated on ice for 5 min in 10 mM EDTA, then filtered through a 40-μm nylon mesh (BD). Single-cell suspensions from either tissue were centrifuged and washed twice with “flow buffer” consisting of HBSS with 2 mM EDTA and 2% FBS.

**Immunostaining and flow cytometry**

Single-cell suspensions were incubated (5 min) with rat and mouse serum (The Jackson Laboratory) containing Ab to CD16/32 (2.4G2; BD Pharmingen), and CD49a (Ha31/8 [BD Pharmingen] and CD49a/α1 integrin directly conjugated to FITC was a gift from D. Topham). Remaining antibodies used were the following (from Biolegend): CD3 (17A2), B220 (RA3-6B2), Ly6C/Ly6G or GR-1 (RB6-8C5), CD11c (N418), Thy 1.2 (30-H12), CD45 (30-F11), CD11b (M1/70; eBioscience), CD11c (HL3; BD Pharmingen), and CD49a (Ha31/8 [BD Pharmingen] and CD49a/α1 integrin directly conjugated to FITC was a gift from D. Topham).

**In vitro detachment and chemotactic migration assays**

Itga1+/+ and Itga1−/− macrophages were isolated from 4-d thioglycollate–treated peritoneal cavities (2 ml, i.p.) and incubated ex vivo for 24 h with LPS (100 ng/ml in RPMI and 10% FBS) to induce α1 integrin expression. Alternatively, peritoneal exudates from 24-h Con A (100 μg in PBS, 2 ml, i.p.)–stimulated mice were used either as mixed populations or enriched for macrophages by adherence to plastic. Macrophages were allowed to adhere to plastic for 30 min at 37°C and were detached using 10 mM EDTA. In both the thioglycollate and enriched Con A methods, macrophages were suspended in HBSS at 106 cells/ml with 2 mg/ml fatty acid–free BSA (Sigma) and 2 mM EDTA on ice after the method of Tcherneychev et al. (17). Macrophages were diluted to 106 cells/ml in HBSS with 2 mg/ml fatty acid–free BSA at 37°C. These cells were immediately introduced into the flow chamber and allowed to adhere for 5 min. Images of four high-powered fields were acquired by fluorescence microscopy; then a shear force of 10 dynes/cm2 was applied for 1 min to induce cell detachment. After flow was stopped, images of four additional high-powered fields were obtained, and macrophages that remained adherent before and after shear were counted. Chemotactic migration assays were performed in Corning Costar Transwell chambers with 5-μm microporous membrane filters separating the upper and lower chambers. Membranes were coated overnight (4°C) with collagen IV (2.5 μg/ml), collagen I (1 μg/ml), laminin (10 μg/ml), or fatty acid–free BSA (10 mg/ml; Sigma), then rinsed with PBS, ECM gel (Sigma; 10 μl used undiluted, “neat”) was applied to membranes for 10 min (22°C) and allowed to polymerize (as per manufacturer’s instructions). Transwell experiments were carried out at 37°C in DMEM with 2 mg/ml fatty acid–free BSA. Positive controls were obtained using iNOS101 Penillin V/Streptomycin (Wisent). Two hundred macrophages were introduced into the upper chamber. For migration experiments, a FACSAria II (BD Biosciences) was used to sort single-cell suspensions from popliteal LN draining 24-h Con A–infused footpads. For sorting, cells were cytopsin onto slides and stained with a Romanowsky stain (Diff-Quick) according to the manufacturer’s instructions.

**Quantitative PCR**

Total RNA from footpads was isolated using the RNeasy Mini plus Isolation Kit (Qiagen). Reverse transcription into single-stranded cDNA was performed using the high-capacity reverse transcription kit (Powerscript from Clontech, Palo Alto, CA), which contains random primers. PCR primers were designed using the Primer Express software (Applied Biosystems). Real-time PCRs were performed using LightCycler 480 detection system (Roche), and amplified DNA was detected by incorporation of SYBR Green (Roche). A standard curve was generated for each primer pair using a mixture of various normal and inflamed tissues. The advanced relative quantification method (LightCycler 480 software) was used to determine the relative gene expression levels in footpads, and data were normalized to both hypoxanthine phosphoribosyltransferase and CD31. The following nucleotide sequences of quantitative PCR primers (forward and reverse, respectively) were used: IFN-γ: 5’-TTGGAATTTGGAAGACACAC-3’; 5’-GGTTCCCTCAGCGCTGTAATCC-3’; IL-1β: 5’-AGTTGACCAGGCACACAAAGA-3’; 5’-TGCTGCTGGAAGGATGAGG-3’; IL-6: 5’-CTCCCAAAGCACGCTCTTAACACCA-3’; 5’-TGCGTATTGGCACTCCTTCTT-3’; TNF-α: 5’-TAGGCGTCACCAGCAAGAGC-3’; 5’-GACGAGCAGAGGCACTCA-3’; hypoxanthine phosphoribosyltransferase: 5’-CAAGGTGTGTTGGAAAAAGGA-3’; 5’-GAAAAGCAGGACAGCTCA-3’; hypoxanthine phosphoribosyltransferase: 5’-CAAGGTGTGTTGGAAAAAGGA-3’; 5’-GAAAAGCAGGACAGCTCA-3’.

**Results**

Macrophones in the inflamed footpad express α1β1 integrin

Our initial aim was to assess the expression of α1β1 integrin on a heterogeneous population of leukocytes in a peripheral inflammatory lesion. We selected the mannan binding lectin Con A, which has previously been shown to induce an inflammatory lesion in the mouse footpads with a mixed population of leukocytes (18). Footpad swelling was serially measured and reached a maximum increase at 24 h (Fig. 1A) consistent with previous studies (19). At 24 h, footpads contained a clearly visible cellular inflammatory lesion. We selected the mannan binding lectin Con A, or pertussis toxin (List Biochemicals). Macrophages were allowed to chemotactically migrate through the coated membranes for 18 h, at which point the cells that had migrated to the lower chamber were isolated, immuno-
FIGURE 1. Macrophages express the highest levels of α1 integrin in the inflamed footpad. (A) Con A–induced swelling reaches a maximum at 24 h. Footpad thickness was measured with a Mitutoyo micrometer, and a time-dependent increase in percent swelling relative to the contralateral control footpad was observed (two independent experiments, n = 5, mean ± SEM). (B–E) Con A induces a highly cellular inflammatory exudate in the mouse footpad. H&E-stained cross sections of the normal (B, C) and 24-h Con A–inflamed footpads (D, E). Dashed boxes in low-magnification (original magnification ×4) views (B, D) indicate the location of the intermediate-magnification (original magnification ×20) views (C, E). Representative images from two independent experiments with n = 4 mice). (F) Flow cytometry of pooled popliteal LNs draining 24-h Con A–inflamed footpads (three independent experiments, n = 9, three LNs from three mice per experiment). Cytospins of sorted cells were based on the indicated gates, and percentages were calculated from the immediately preceding parent gates. Representative images of cytospins: macrophages (Macs), eosinophils (Eos), and neutrophils (Neuts). Scale bars, 20 μm. (G) Flow cytometry of digested 24-h Con A–inflamed footpads gated on total CD45+ cells showing α1β1 integrin expression (Figure legend continues).
Cell suspensions from digested footpads and draining popliteal LNs were stained for multiple cell-surface markers and analyzed by flow cytometry. A combination of markers including the GR-1 Ab, CD11b, and side-scatter signal allowed us to identify specific myeloid subsets (20) (Fig. 1B). Cells were sorted and cytospins performed to validate this gating strategy, and we could clearly identify macrophages, eosinophils, and neutrophils microscopically (Fig. 1F). Analysis of CD45+ cells in footpad single-cell suspensions revealed that macrophages expressed the highest levels of α1β1 integrin (Fig. 1G). We also found that blood monocytes and macrophages in normal and inflamed LNs preferentially express α1β1 integrin (Supplemental Fig. 1). Prior studies established the expression of α1β1 integrin on T cells in chronic inflammatory lesions and in vitro on macrophages (13, 16). Our data establish that in vivo monocytes express α1β1 integrin in the blood, and macrophages express α1β1 integrin in an acute inflammatory lesion and the draining LN.

Exit of leukocytes from the inflamed footpad can be tracked by ISPL using the fluorescent dye CFSE

In large animals, it has been possible to assess the exit of endogenous leukocytes from peripheral tissues by surgical cannulation of afferent lymphatics (21–23). In murine models, data on leukocyte exit from tissues have been generated by adoptive transfer experiments including s.c. injection of leukocytes into footpads or intratracheal delivery into the lungs (24–27); studies transferring leukocytes into mouse footpads have established the draining popliteal LN as their first destination, where they accumulate over time.

Analogous to these adoptive transfer experiments, we sought to assay the exit of endogenous leukocytes from the inflamed mouse footpad to the draining popliteal LN. We developed an ISPL technique to fluorescently label endogenous leukocytes exclusively in the inflamed footpad with CFSE (Fig. 2A). This technique avoids spillover of dye and resultant “bystander” labeling of the draining LN by transient occlusion of the draining lymphatic vessels. Dye spillover and “bystander” labeling of the draining LN would have made it impossible to determine from where CFSE+ cells originated. CFSE is highly labile in aqueous environments; thus, rapid deactivation occurs in aqueous solutions in vitro and in tissues in vivo (28, 29). Within 5 min after footpad injection, CFSE is rendered inert and unable to effectively label cells. Over time, however, CFSE+ cells migrate via afferent lymphatics and accumulate in the draining LN.

We analyzed footpads and their draining popliteal LNs 15 min after ISPL. Approximately 70% of leukocytes in the excised footpad tissue were CFSE+, whereas the draining popliteal LN was devoid of CFSE+ cells (Fig. 2B). CFSE+ leukocytes could be detected 4 h after ISPL in the draining popliteal LN, and the percentage of CFSE+ cells decreases in the inflamed footpad from 15 min to 4 h (Fig. 2B). This is because some of the CFSE+ cells exit the footpad and migrate to the draining LN. Furthermore, unlabeled (CFSE−) leukocytes are recruited from the blood to the Con A–inflamed footpad during the experiment and over time, these cells significantly outnumber the CFSE+ population that remains in the footpad tissue.

A time course was performed to characterize the exit of leukocytes from the inflamed footpad. This showed that maximal LN accumulation of CF45+CFSE+ leukocytes occurred between 4 and 8 h after ISPL (Fig. 2C, 2D). The percentage of CFSE+ cells in the LN is relatively small because the LN has vastly more leukocytes than the footpad, and the LN consists of entirely CFSE− cells. Previous studies using s.c. injected lymphocytes have shown that beyond 16 h, cells can be found in the circulation (26). Thus, at 4 and 8 h, the CFSE+ leukocytes had migrated directly from the inflamed footpad and reached the popliteal LN via afferent lymphatics, but had not yet entered the circulation. Based on these data and prior studies showing that in vivo CFSE stabilizes by 4 h (28), we chose the 4-h time point for subsequent investigations. Experiments with pertussis toxin inhibited the accumulation of CFSE+ leukocytes in draining popliteal LN and confirmed that CFSE dye does not diffuse out of the footpad and label cells in the node (Supplemental Fig. 2). In summary, we developed a physiological bioassay in which ISPL fluorescently labels a “cohort” of endogenous leukocytes in the inflamed footpad. These CFSE+ cells progressively exit the footpad and accumulate in the draining LN where they can be isolated and analyzed by flow cytometry.

Igα1−/− macrophages display accelerated exit from the inflamed footpad

We investigated the effect of genetic deletion of α1β1 integrin and how this affected leukocyte migration from the inflamed footpad to the draining LN. Inflamed footpads were treated with ISPL at 24 h, and 4 h later, draining popliteal LNs were isolated (Fig. 3A). LNs were then analyzed for CFSE+ cells of each subtype, and data were normalized to wild type Igα1+/− migration (Fig. 3B, 3C). Both Igα1+/− and Igα1−/− CFSE+ macrophages were found in draining popliteal LNs. However, CFSE+ macrophages consistently displayed more migration in Igα1+/− mice relative to Igα1+/− mice (Fig. 3C). In contrast, the migration of other CFSE+ leukocyte subsets was similar in the draining popliteal LNs (Fig. 3C). These data suggested the possibility that Igα1−/− macrophages migrated more efficiently from the inflamed footpad and this could account for their greater percentage in the draining LN. We hypothesized that α1β1 integrin–mediated adhesion was “anchoring” macrophages in the inflamed footpad, effectively inhibiting their exit.

We also considered alternative mechanisms that could have explained our results. Collagen IV is the principal ligand for α1 integrin, and collagen IV is highly expressed in basement membranes. To verify that these well-known expression patterns were similar in both Igα1+/− and Igα1+/− mice, we stained histological sections for basement membranes and collagen IV (Supplemental Fig. 3). Similar expression patterns of basement membrane, as well as abundance of collagen IV, were seen in both Igα1+/− and Igα1+/− mice (Supplemental Fig. 3). Another consideration we had was that, in theory, a different collagen-binding integrin could potentially “compensate” for α1 integrin deficiency. The collagen I–binding integrin α2 is one possibility because collagen I is plentiful in extravascular tissues. We stained for α2 integrin in the inflamed LN and found that expression levels were comparable in Igα1+/− and Igα1−/− mice (Supplemental Fig. 4A). This establishes that collagen IV expression is similar between the genotypes, as is expression of an alternate collagen receptor α2 integrin. Thus, these possible alternative mechanisms cannot explain our results.

To rule out the possibility that there were differences in the inflammatory responses between Igα1+/− and Igα1−/− mice, we analyzed several parameters of inflammation and at 24 h, the inflammatory responses of these two genotypes were similar. There were no statistical differences in comparing the relative abundance (white histograms). The negative control (gray) is based on footpad cells from Con A–inflamed Igα1−/− mice using the same staining protocol. Representative staining of digested footpads from four independent experiments with n = 4 mice.
of common proinflammatory cytokine mRNAs, the absolute number of cells in the draining popliteal LNs, footpad swelling, or histological appearance (Fig. 3D–G). Thus, we conclude that the observed difference in macrophage migration is unlikely due to differences in inflammatory responses between the two genotypes.

If Itga1−/− macrophages exited more efficiently from the inflamed footpad, this could influence the resolution of inflammation. Con A induces an acute inflammatory response and swelling in the footpad that mostly resolves by 48 h (Fig. 1A). Nevertheless, we compared the relative abundance of proinflammatory cytokine mRNAs in Itga1+/+ and Itga1−/− mice at 48 h, in addition to our 24-h data (Supplemental Fig. 4B). In contrast with the 24-h time point (Fig. 3D), we found that IL-1β and TNF-α mRNA levels were reduced in Itga1−/− mice relative to wild type mice. This is consistent with the possibility that more efficient exit of macrophages from the site of inflammation leads to reduced local proinflammatory cytokine production and accelerated resolution of inflammation.

**Blockade of α1β1 integrin results in accelerated macrophage exit from the inflamed footpad**

If adhesion via α1β1 integrin was retaining macrophages in the inflamed footpad, then disrupting its adhesive function should result in more macrophages exiting the footpad over time. Mouse footpads were inflamed with Con A and 24 h later, neutralizing Ab to α1β1 integrin was injected directly into the footpad, contemporaneously with ISPL (Fig. 4A). We compared this with control IgG injections using the same protocol (Fig. 4A), and performed flow cytometry on single-cell suspensions from the draining LNs (Fig. 4B). Over a 4-h period, draining popliteal LNs were enriched for CFSE+ macrophages by ∼2-fold when footpads were treated with α1β1 blocking Ab as compared with control IgG (Fig. 4B, 4C). Blockade of α1β1 integrin had no effect on other CFSE+ leukocyte subsets (Fig. 4C).

Although consistent with our hypothesis, these results could potentially be attributed to treatment effects on the draining popliteal LN. It is assumed that CFSE+ leukocyte accumulation in the popliteal LN is consistent across different treatments. However, Ab injected into the footpad would reach the draining LN and could potentially affect leukocyte accumulation. To address this possibility, we injected Evans blue dye directly into the footpad lesion and compared this with a calf injection (distal calf). When injected into the calf, Evans blue dye could not be detected in the inflamed footpad lesion (Fig. 4D), but calf injections still reach the draining popliteal LN (20). Therefore, we used ISPL and injected α1β1 neutralizing Ab into the calf, outside of the inflammatory lesion (Fig. 4C). Results show that calf injections of α1β1 integrin neutralizing Ab have no effect on macrophage accumulation in the draining popliteal LN (Fig. 4C). The Ab blockade only enhances macrophage exit if delivered directly into the inflamed footpad.

Given the bivalent nature of IgG molecules, it was possible that the blocking Ab was cross-linking α1β1 integrin, which could potentially induce “outside-in” signaling. The increased accumulation of macrophages in the popliteal LNs of mice treated with neutralizing Ab could be attributed to integrin cross-linking and signaling, rather than a blockade of adhesion. To control for the possible effects of integrin cross-linking, we sought to perform a monovalent blockade of α1β1 integrin. To this end, we sought out a small-molecule inhibitor of α1β1 integrin adhesion. Small-molecule integrin inhibitors have been described and are known as disintegrins, some of which are derived from viper venom.

Proteins from viper venom have been shown to have a wide variety of biological effects, some of which are specific to integrins (30). The disintegrin Obtustatin, from the blunt-nosed viper Vipera lebetina obtusa, is one such molecule. Obtustatin is a 41-aa monovalent disintegrin (31), and it has been shown to block the
adhesion of αβ1 integrin to collagen IV with high specificity. Because Obtustatin interacts with αβ1 integrin monovalently, we could perform a functional blockade without cross-linking the integrin (31).

We initiated a blockade of αβ1 integrin in the inflamed mouse footpads using Obtustatin or its inactivated form ethylpyridylated-Obtustatin (EP-Obtustatin) as a control (31). Similar to the Ab blockade, Obtustatin treatment resulted in a modest but reproducible enrichment of CFSE+ macrophages in the draining LN when compared with controls (Fig. 4E). Consistent with the Ab treatments, increased migration was only seen in the macrophage population (Fig. 4E). Given these results and the monovalent nature of Obtustatin, it is unlikely that the altered macrophage migration was due to “outside-in” signaling triggered by integrin cross-linking caused by Ab treatment. These data reinforce the hypothesis that αβ1 integrin specifically mediates macrophage adhesion and retention in the inflamed footpad, inhibiting macrophage exit.

Adhesion to collagen IV reduces macrophage chemotactic migration efficiency

We next tested the adhesive function of αβ1 integrin in vitro. Previous studies have shown that collagen IV is a relatively specific ECM ligand for αβ1 integrin, and that embryonic fibroblasts and T cells from Itgal−/− mice exhibit reduced adhesion to collagen IV (13, 14, 31). Macrophages in the inflamed footpad express relatively high levels of αβ1 integrin compared with other cell types (Fig. 1C), but whether macrophages also use αβ1 integrin to adhere to collagen IV is unknown. We therefore performed in vitro detachment assays to competitively assess the relative strength of adherence between Itgal+/+ and Itgal−/− macrophages.

Macrophages were harvested from inflamed peritoneal cavities of Itgal+/+ and Itgal−/− mice, differentially labeled in vitro with either CFSE or CMPTX (Cell tracker red), then admixed in approximate equal numbers (Fig. 5A). These macrophages were introduced into a parallel plate flow chamber system coated with collagen IV and allowed to adhere (Fig. 5A). After exposure to a detaching shear force of 10 dynes/cm², an average of ~56% more Itgal+/+ macrophages remained adherent to the collagen IV–coated plates compared with Itgal−/− macrophages (Fig. 5B). Pretreatment of macrophages with either αβ1 integrin blocking Ab or Obtustatin eliminated this difference (Fig. 5B). These results provide direct evidence that Itgal−/− macrophages have a relative deficiency in adhesion to collagen IV and that αβ1 integrin mediates this adhesion.

To investigate the role of αβ1 integrin–mediated adhesion in the context of migration, we performed chemotaxis assays.
Transwell microporous membranes were coated with either control BSA or different ECM molecules: collagen IV, collagen I, laminin, or an ECM gel containing multiple ECM molecules including 70% collagen IV. Peritoneal macrophages from Itga1+/+ and Itga12/2 mice were differentially labeled, admixed, and placed in the upper chamber; the lower chamber contained the chemokine CCL21, creating a chemotactic gradient to drive migration (Fig. 5C).

Consistent with our prediction, when membranes were coated with collagen IV–containing substrates, Itga1+/+ macrophage migration was less efficient compared with Itga12/2 macrophages (Fig. 5C). However, there was no difference in macrophage migration efficiency across substrates that lacked collagen IV such as BSA, collagen I, or laminin (Fig. 5C). These data support the hypothesis that macrophages expressing α1β1 integrin adhere to collagen IV and this decreases their chemotactic migration efficiency.

**Discussion**

Leukocytes at inflammatory lesions constitute an important component of the immune response. The accumulation and persistence of leukocytes in inflamed tissues is regulated by multiple mechanisms including the balance between leukocyte entry from the blood and leukocyte exit to afferent lymphatics and the draining LN (32). Leukocyte exit from peripheral tissues relies on the expression of G protein–coupled chemokine receptors such as CCR7 and sphingosine-1 phosphate (S1P) receptor, the S1P receptor having originally been discovered as crucial for lymphocyte exit from secondary lymphoid organs (24, 26, 27, 33). In contrast, leukocyte entry into tissues from the blood is governed by a multistep cascade that is mediated by a series of adhesion molecules including integrins (2, 34). The adhesive role of integrins in the blood to tissue leukocyte migration is well established. The objective of this study was to investigate the potential role of integrin-mediated adhesive mechanisms in controlling leukocyte exit from a peripheral inflammatory lesion.

Our data demonstrate a role for integrin-mediated adhesion of macrophages in inflamed tissues, specifically the collagen IV–binding integrin α1β1. To our surprise, rather than functioning in a promigratory capacity, α1β1 integrin inhibited macrophage exit from a peripheral inflammatory lesion. Staining of the inflamed mouse footpad cells revealed that macrophages expressed the highest levels of α1β1 integrin (Fig. 1G). Consistent with this staining pattern, α1β1 integrin blockade or genetic deletion affected the exit of macrophages from the inflammatory lesion, but not other leukocytes. We postulate that α1β1 integrin “anchors” macrophages in the inflamed footpad, inhibiting their exit. To our knowledge, this is the first study to directly demonstrate the migration-inhibiting function of a leukocyte integrin from a peripheral inflammatory lesion.

We present a novel experimental technique that we have called ISPL. Using the fluorescent dye CFSE, ISPL exclusively labels...
a cohort of endogenous leukocytes in the inflamed footpad, but the draining LN remains unlabeled (Fig. 2B). Over time, leukocytes exit the inflamed footpad via afferent lymphatics and accumulate in the popliteal LN. Accumulated CFSE+ cells in the LN can then be isolated and identified by flow cytometry. The major advantage of our approach is the labeling of endogenous leukocyte populations in situ in mice. This is a significant advance because leukocytes in inflamed tissues may represent unique populations with specific localizations in the tissue microenvironment. In addition, the endogenous distribution of leukocytes may be important for their access to adhesive and chemotactic ligands. It is our hope that ISPL will complement other approaches and help expand the repertoire of useful in vivo methods.

During the process of choosing an inflammatory stimulus, we considered several important factors including effective recruitment of a heterogeneous population of leukocytes and induction of comparable acute inflammatory responses in both Itga1−/− mice and Itga1+/− mice to enable comparisons between these genotypes. Because Itga1−/− mice or mice systemically perfused with α1β1 integrin blocking Ab have reduced T cell–mediated immune responses (15, 16, 35–37), we considered T cell–independent inflammatory stimuli. Initially, we used TLR ligands such as LPS and polyinosinic-polycytidylic acid. However, we found that TLR ligands induced neutrophil-dominated responses, consistent with previously published data (38–40). This led us to select the mannose binding lectin Con A. Con A–induced robust inflammation and lesions contained a heterogeneous population of inflammatory cells including a broad range of different leukocyte subsets (18, 19) (Figs. 1B–G, 3G). Moreover, there were no significant differences in the inflammatory responses between Itga1−/− and Itga1+/− mice, suggesting the effect on macrophages was not due to differences in inflammation (Fig. 3D–G). Thus, the Con A inflammatory reaction allowed us to analyze, compare, and contrast a mixed population of inflammatory leukocytes exiting the footpad.

We specifically tested macrophage adhesion and migration to collagen IV substrates in vitro. Collagen IV is a specific ligand for α1β1 integrin (11). Wild-type macrophage adhesion to collagen IV was strengthened via α1β1 integrin relative to Itga1−/− macrophages; blocking of α1β1 integrin abolished this adhesive advantage (Fig. 5A, 5B). The function of α1β1 integrin was also tested in transwell chemotaxis experiments. Macrophages migrating to the CCR7 ligand CCL21, but transwell membranes coated with substrates containing collagen IV inhibited wild type macrophage migration (Fig. 5C). Wild-type macrophage migration efficiency could be reduced to as low as ~25% relative to Itga1−/− macrophages, and this was achieved by coating transwell membranes with ECM gel (~70% collagen IV; Fig. 5C). The ECM gel was visibly thicker than other coatings, and we speculate that this provided more ligand binding sites and thus a greater opportunity for adhesion via α1β1 integrin. These data show that macrophage chemotactic migration can be inhibited by α1β1 integrin binding to collagen IV.

The α1β1 integrin is important for initiating inflammation, as well as the maintenance of chronic inflammatory responses. Mice genetically deficient in α1β1 integrin or treated with systemic α1β1 integrin neutralizing Ab show decreased inflammation in the effector phase of T cell–mediated responses such as delayed-type hypersensitivity, chronic decreased inflammation, and decreased resistance to viral infection (12, 13, 15, 35, 37). Intriguingly, reduced numbers of T cells were found at inflammatory lesions in Itga1−/− mice or mice treated with α1β1 integrin neutralizing Ab, and it was postulated that α1β1 integrin was “retaining” T cells at these inflammatory sites (12, 15, 35). This attractive hypothesis is consistent with our results showing that α1β1 integrin inhibits macrophage exit from the inflamed footpad. Subsequent studies showed that T cell–dependent inflammatory responses required T cell–macrophage interaction for effective macrophage production of proinflammatory cytokines (16). This novel macrophage activation pathway depends on the direct binding of Sema7A on T cells to α1β1 integrin on macrophages. Notably, both Sema7A−/− and Itga1−/− mice are deficient in the effector phase of T cell–mediated inflammatory responses establishing an in vivo role for these molecules (13, 16). Macrophages are critical for peripheral inflammatory responses, and their presence or absence can have dramatic effects (41, 42). Given that the persistence of macrophages in tissues and that the cytokines they secrete are important in the maintenance of inflammatory responses, we speculate that more rapid macrophage exit could lead to decreased inflammation. This implies that control of macrophage exit from tissues could represent an additional regulatory mechanism for inflammatory processes. However, whether α1β1 integrin can modulate these responses via macrophage persistence or exit from inflamed tissues remains to be elucidated.

Integrins involved in blood-to-tissue migration, such as α4β1 or αLβ2 (LFA-1), are constitutively expressed on particular leukocyte subsets and, in fact, require a chemokine receptor–mediated
activation step for effective adhesion (7, 34). In contrast, in T cells α1β1 integrin generally requires an inflammatory stimulus to be expressed and to date has been found predominantly in extra-
vascular tissues (15, 43). Moreover, unlike α4β1 or α6β2, genetic deletion or blockade of α1β1 integrin has no effect on blood-to-
tissue leukocyte recruitment (12, 15). In extravascular tissues, leukocyte exit is mediated by chemokine receptors such as CCR7 (24, 26), as well as SIP receptor (27). Leukocytes are chemo-
tactically attracted to afferent lymphatics that express the CCR7 ligand CCL21 (44). CCR7-dependent tissue exit has been ob-
served in both homeostatic and inflammatory conditions (24, 26).

In contrast, we demonstrate that α1β1 integrin adhesion inhibits macrophage exit from a peripheral inflammatory lesion (Figs. 3C, 4C, 4E). We speculate that there could be an antagonistic re-
relationship between α1β1 integrin and chemokine receptors such as CCR7. This implies that balancing these opposing forces would be important in determining the extent of exit or persistence of macrophages in tissues; however, this remains to be investigated.

Based on our studies, we speculate that other integrins might function to inhibit exit of leukocytes from the periphery in a manner similar to α1β1 integrin. ECM binding integrins could be con-
sidered prime candidates and include α2β1, α5β1 α6β1, and αvβ3 (7). The inflammatory state of the tissue milieu is also likely to be important and could affect both the expression patterns and function of leukocyte integrins.

In conclusion, we have demonstrated a new role for leukocyte integrins in inflamed extravascular tissues. In addition to the well-
described promigratory role of integrins in the blood-to-tissue paradigm, we show that α1β1 integrin adhesion specifically in-
hibits macrophage exit from a peripheral inflammatory lesion. Given the important functions of macrophages as multifunctional phagocytes involved in the initiation, maintenance, and resolution of inflammation (41, 42, 45–47), the migration-inhibiting function of α1β1 integrin may be considered significant. Our data identify α1β1 integrin as a possible target for regulating macrophage mig-

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Disclosures

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