Importance of integrin LFA-1 deactivation for the generation of immune responses

Monika Semmrich,1 Andrew Smith,3 Carolin Feterowski,1 Sandra Beer,1,4 Britta Engelhardt,5 Dirk H. Busch,2 Bernadett Bartsch,1 Melanie Laschinger,3 Nancy Hogg,1 Klaus Pfeffer,4 and Bernhard Holzmann1

The online version of this article contains supplemental material.

The dynamic regulation of ligand binding is considered crucial for integrin function. However, the importance of activity regulation for integrin function in vivo is largely unknown. Here, we have applied gene targeting to delete the GFFKR sequence of the lymphocyte function-associated antigen–1 (LFA-1) α subunit cytoplasmic domain in mouse germline. Lymphocytes from Lfa–1−/− mutant mice showed constitutive activation of LFA-1–mediated cell adhesion and impaired de-adhesion from intercellular adhesion molecule–1 that resulted in defective cell migration. In contrast, signaling through LFA-1 was not affected in Lfa–1+/− cells. T cell activation by superantigen-loaded and allogeneic APCs, cytotoxic T cell activity, T-dependent humoral immune responses, and neutrophil recruitment during aseptic peritonitis were impaired in Lfa–1−/− mice. Thus, deactivation of LFA-1 and disassembly of LFA-1–mediated cell contacts seem to be vital for the generation of normal immune responses.

Many integrins do not exhibit constitutive ligand binding capacity, but are expressed in an inactive state (1, 2). Integrin ligand binding is activated rapidly and transiently by cytoplasmic signals initiated by the stimulation of diverse cell surface receptors (inside-out signaling). The integrin, lymphocyte function-associated antigen–1 (LFA-1; αβL), is expressed on all leukocytes and is considered to play an important role in host defense (3). LFA-1 shares the β2 subunit with Mac-1 (αMβ2), p150/95 (αMβ2), and αβL. Binding of LFA-1 to its natural ligands, intercellular adhesion molecule (ICAM)–1, ICAM–2, or ICAM–3, mediates interactions between T cells and blood vessel endothelium, target cells, and APCs. LFA-1 stabilizes intercellular adhesion between T cells and APCs, which seems to be particularly relevant for facilitating T cell activation at low antigen concentration (4). In addition, binding of LFA-1 to its cognate ligands directly activates the Jnk and Erk–1/2 pathways, and thereby decreases the threshold of T cell activation (5, 6). LFA-1–deficient mice exhibit reduced lymph node cellularity and splenomegaly which is consistent with a major role of LFA-1 in normal lymphocyte recirculation (7–10). T cells from LFA-1−/− mice also displayed an impaired proliferation in response to allogeneic cells and mitogen. However, they mounted normal cytotoxic T cell responses during viral infections, but failed to reject immunogenic tumors (7).

The ligand-binding capacity of leukocyte integrins can be activated by signaling pathways emanating from T and B cell antigen receptors, chemokine receptors, and Toll-like receptors within a time-scale of seconds to minutes (1, 11–13). Integrin activation through these receptors causes large-scale conformational changes in integrin extracellular domains that are associated with increased ligand-binding affinity and leads to receptor clustering at cell contact areas. The signaling pathways that control LFA-1 activation are not understood completely, but involve protein kinase C; phosphatidylinositol 3-kinase; cytohesin–1; small GTPases; and the adaptor protein, SLAP–130/Fyb (14–20).

The cytoplasmic domains of α and β subunits are crucial for the regulation of integrin...
ligand-binding activity (1). Thus, changes in the cytoplasmic tails of integrins affect the structure and function of their extracellular domains. Mutational analyses have shown that interactions between membrane-proximal sequences of integrin cytoplasmic domains restrain integrins in an inactive conformation. For example, deletions of the entire α subunit cytoplasmic domain or of the highly conserved membrane-proximal GFFKR sequence disrupt interactions of α and β subunit tails, and produce constitutively active integrin in vitro. Consistent with this model, the talin head domain activates integrins by binding to the cytoplasmic domain of β subunits and—as a result of the high affinity of this interaction—ablates interactions between α and β cytoplasmic domains. Recent studies that measured fluorescence resonance energy transfer confirmed these changes of cytoplasmic domain interactions during integrin activation in living cells in vitro (21).

Although numerous studies have investigated the consequences of integrin deficiencies in mouse models, the in vivo relevance of restraining LFA-1 in an inactive state on resting leukocytes and rapidly deactivating LFA-1 after cell stimulation is unknown. In the present study, we mutated the α, subunit cytoplasmic tail in mouse germline leading to the expression of constitutively active LFA-1. We show that this mutation severely impairs immune responses in vitro and in vivo, and thereby demonstrate that complete deactivation is essential for the normal function of LFA-1.

Figure 1. Generation of Lfa−1mutant mice. (a) Organization of the murine Lfa−1 genomic locus (top), targeting vector (second from top), targeted Lfa−1 allele (second from bottom), and Lfa1 allele following intercross with the cre deleter strain (bottom). (b) Southern blot analysis of genomic DNA derived from a wild-type mouse (lane 1) and a heterozygous Lfa−1/neu mouse (lane 2) before deletion of the neocassette using the HpaI probe. (c) PCR analysis of genotypes from wild-type (+/+) and homozygous Lfa−1 (d/d) mice after deletion of the neocassette. To detect the mutant allele, a genomic fragment containing the newly introduced HpaI site in exon 31 was amplified by PCR and digested with HpaI.
RESULTS

Generation of a mutant mouse strain (Lfa-1\(^{d/d}\)) expressing constitutively active integrin LFA-1

Although the lack of LFA-1–mediated cell adhesion has been demonstrated to impair immune responses (7), the functional consequences of defective LFA-1 deactivation are unknown. To address this question, a mutant mouse strain (Lfa-1\(^{d/d}\)) that exhibits a constitutive germline deletion of the conserved amino acid motif GFFKR in the membrane-proximal segment of the LFA-1 \(\alpha_L\) chain cytoplasmic domain was generated by gene targeting (Fig. 1). This mutation was chosen because deletion of the GFFKR sequence is known to cause separation of the \(\alpha_L\) and \(\beta_2\) cytoplasmic tails, and to result in the constitutive activation of integrins, including LFA-1 (21–23). Correct introduction of the GFFKR deletion was confirmed by cDNA cloning and nucleotide sequence analysis of \(\alpha_L\) transmembrane and cytoplasmic domains from Lfa-1\(^{d/d}\) mice (unpublished data). Mutant Lfa-1\(^{d/d}\) mice were born at normal Mendelian ratios, were fertile, and did not exhibit any gross anatomic or behavioral abnormalities (unpublished data).

To control whether the Lfa-1\(^{d/d}\) mutation may affect integrin expression, flow cytometry analyses were performed. The results in Fig. 2 a show that cell surface expression of mutant LFA-1 was reduced as compared with the wild-type integrin. However, analysis of permeabilized cells revealed that the levels of total cellular LFA-1 protein were not altered by the Lfa-1\(^{d/d}\) mutation (Fig. 2 a). Moreover, splenocyte mRNA levels for mutant and wild-type LFA-1 did not differ significantly (unpublished data), and indicated that expression of the mutant Lfa-1\(^d\) gene was not impaired. Instead, these findings are consistent with previous observations showing that deletion of the membrane-proximal GFFKR sequence in the LFA-1 \(\alpha_L\) subunit affects surface expression by reducing heterodimer formation with the \(\beta_2\) subunit (23). Additional flow cytometry analyses of B and T cells and neutrophils demonstrated that the integrins, \(\alpha_L\beta_1\) and Mac-1, which share the \(\beta_2\) subunit with LFA-1, were expressed at similar levels on Lfa-1\(^{d/d}\) and wild-type cells (Fig. 2 b). Moreover, Lfa-1\(^{d/d}\) and wild-type cells also did not differ in their expression of unrelated \(\alpha_i\beta_j\) integrins (Fig. 2 b); this indicates that the Lfa-1\(^{d/d}\) mutation does not alter the global expression of leukocyte integrins.

The effect of the Lfa-1\(^{d/d}\) mutation on cell adhesion was investigated using a recombinant ICAM-1(D1-2)-Fc protein. The results in Fig. 3 a demonstrate that adhesion of unstimulated Lfa-1\(^{d/d}\) thymocytes to ICAM-1(D1-2)-Fc was significantly elevated as compared with cells that expressed wild-type LFA-1. Exposure to the integrin activator, Mn\(^{2+}\), or incubation with phorbol ester further increased binding of Lfa-1\(^{d/d}\) and wild-type thymocytes to ICAM-1(D1-2)-Fc. However, even under these conditions, Lfa-1\(^{d/d}\) cells adhered more strongly to ICAM-1(D1-2)-Fc than did wild-type cells. Notably, ICAM-1(D1-2)-Fc adhesion of unstimulated Lfa-1\(^{d/d}\) cells was similar to that of Mn\(^{2+}\) or PMA-stimulated wild-type cells (Fig. 3 a). Maximal adhesion of wild-type thymocytes required stimulation with Mn\(^{2+}\) and PMA, whereas for Lfa-1\(^{d/d}\) cells, incubation with a single stimulus was sufficient. The absolute levels of maximal adhesion to ICAM-1(D1-2)-Fc did not differ between Lfa-1\(^{d/d}\)

---

**Figure 2. Integrin expression in Lfa-1\(^{d/d}\) mice.** (a) Cell surface levels of LFA-1 and total cellular LFA-1 protein were measured by flow cytometry analyses of Lfa-1\(^{d/d}\) (red lines) and wild-type (blue lines) splenocytes. (b) Cell surface integrin expression was determined on Lfa-1\(^{d/d}\) (red lines) and wild-type (blue lines) splenic T cells (CD3\(^+\) cells), B cells (B220\(^+\) cells), and bone marrow neutrophils (Gr-1\(^+\) cells). Staining with isotype-matched controls is indicated as dotted lines (n = 3 independent experiments).
and wild-type cells (Fig. 3 a). To control whether the GFFKR deletion in the mouse $\alpha_L$ gene specifically affects the integrin, LFA-1, thymocyte adhesion to vascular cell adhesion molecule (VCAM)-1-Fc, which interacts with $\beta_2^1$ and $\beta_2^7$ integrins, was examined. As depicted in Fig. 3 b, binding of Lfa-1$^{d/d}$ and wild-type thymocytes to VCAM-1-Fc was comparable for all conditions tested. These results demonstrate that the Lfa-1$^{d/d}$ mutation results in a constitutive, but partial, activation of LFA-1. Moreover, the maximal LFA-1–mediated adhesive capacity of cells was not altered by this mutation, and suggests that cell surface expression of mutant LFA-1 is sufficient for its functionality. Consistent with this notion, previous work showed that only a small subset of the integrin, Mac-1 ($\alpha_M^2$), is sufficient to mediate the full adhesive capacity of neutrophils (24).

In contrast to stimulation with PMA, activation of integrin ligand-binding by the T cell antigen receptor and chemokines is mediated by Rap1, together with other signaling molecules (14, 25, 26). The critical cytoplasmic region of $\alpha_L$ that is required for Rap1 responsiveness is adjacent to the GFFKR sequence (27); this raises the possibility that deletion of the GFFKR sequence in Lfa-1$^{d/d}$ mice would alter activation of LFA-1 through the T cell receptor. Therefore, we investigated LFA-1 activation in response to T cell receptor ligation. Lymph node cells from wild-type and Lfa-1$^{d/d}$ mice that were stimulated with PMA and ionomycin, and maintained in IL-2, were stimulated with CD3 antibodies and adhesion to ICAM-1(D1-2)-Fc was measured. The results in Fig. 3 c show that CD3 treatment induced adhesion of wild-type and Lfa-1$^{d/d}$ cells to a similar extent; the adhesion of Lfa-1$^{d/d}$ cells was accelerated slightly 10 min after addition of CD3 antibodies. Therefore, these data indicate that the $\alpha_L$-GFFKR deletion did not impair activation of LFA-1 in response to T cell receptor ligation.

LFA-1 is crucial for the firm adhesion of leukocytes to endothelial cells and the subsequent process of transendothelial migration. Therefore, we investigated the effects of the Lfa-1$^{d/d}$ mutation on leukocyte endothelial interactions. Fig. 3 d shows that binding of resting splenocytes and lymph node cells to unstimulated bEnd5 endothelioma cells was not influenced by the mutation, and suggests that cell surface expression of mutant LFA-1 is sufficient for its functionality. Consistent with this notion, previous work showed that only a small subset of the integrin, Mac-1 ($\alpha_M^2$), is sufficient to mediate the full adhesive capacity of neutrophils (24).

In marked contrast to cell adhesion, Lfa-1$^{d/d}$ lymphocytes that were derived from spleen or lymph nodes were impaired significantly in their capacity to transmigrate bEnd5 endothelial cell monolayers (Fig. 3 e).

### Impaired de-adhesion and migration of lymphocytes expressing mutant LFA-1

To investigate further why the $\alpha_L$-GFFKR deletion conferred enhanced ability of T cells to adhere to ICAM-1, but retarded their ability to migrate across stimulated endothelial cells, we performed additional experiments. LFA-1$^{d/d}$ lymphocytes were stimulated as compared with unstimulated cells. (d) Splenocytes (SPL) or lymph node (LN) cells were plated on bEnd5 endothelioma cells that were unstimulated (medium) or treated with TNF for 16 h. The number of cells adherent per unit area were determined ($n = 5$ independent experiments). (e) Transmigration of unstimulated (medium) or TNF-treated bEnd5 endothelioma cells by splenocytes or lymph node cells (right panel) was measured ($n = 6$ independent experiments). *$P < 0.05$; **$P < 0.01$ (Student’s t test).
The speed of... migration. Thus, complete deactivation of the integrin, LFA-1, mutation results in impaired de-adhesion at trailing edges of T cells. Tracking of individual T cells revealed that the distance migrated by T cells slightly as compared with wild-type cells (Fig. 4, a and b). The average migratory speed of T cells was determined after 30 min (mean ± SD). T cells migrating on 50 μg/ml immobilized ICAM-1-Fc with calculation of speed over 10 min. The speed of Lfa-1<sup>d/d</sup> T cells is expressed as percentage of wt T cell speed ± SEM (**P < 0.01). (c) Phase images of migrating T cells. The red circle indicates the position of the T cell at 0 s; the yellow line details the T cell trajectory over 180 s. White bars, 10 μm. (d) Migration of T cells was tracked over 600 s; each line represents one T cell (wt, n = 20; Lfa-1<sup>d/d</sup>, n = 21). Experiments are representative of n = 2 (a) and n = 6 (b–d).

![Figure 4. T cell adhesion and migration on immobilized ICAM-1–Fc.](image)

(a) Adhesion of spleen T cells onto immobilized ICAM-1 in the presence of 6 mM Mg<sup>2+</sup> was determined after 30 min (mean ± SD). (b) T cells migrating on 50 μg/ml immobilized ICAM-1–Fc with calculation of speed over 10 min. The speed of Lfa-1<sup>d/d</sup> T cells is expressed as percentage of wt T cell speed ± SEM (**P < 0.01). (c) Phase images of migrating T cells. The red circle indicates the position of the T cell at 0 s; the yellow line details the T cell trajectory over 180 s. White bars, 10 μm. (d) Migration of T cells was tracked over 600 s; each line represents one T cell (wt, n = 20; Lfa-1<sup>d/d</sup>, n = 21). Experiments are representative of n = 2 (a) and n = 6 (b–d).

LFA-1 deficiency was found to alter the cellularity of peripheral lymphoid organs (7, 9). Therefore, we investigated the influence of constitutive LFA-1 activation on lymphoid organogenesis. Analysis of different lymphoid organs revealed that total cell numbers in peripheral lymph nodes and Peyer’s patches were reduced substantially in Lfa-1<sup>d/d</sup> mutant mice when compared with wild-type littermates (Table I). In contrast, spleen cell numbers were significantly greater in Lfa-1<sup>d/d</sup> mice than in wild-type mice. Bone marrow and thymus cellularity as well as peripheral blood leukocyte numbers were not altered by the Lfa-1<sup>d/d</sup> mutation. This phenotype of Lfa-1<sup>d/d</sup> mice is similar to that of Lfa-1<sup>−/−</sup> mice, and suggests that the presence and the full conformational flexibility of LFA-1 are required for the normal development of secondary lymphoid organs.

**Table I. Lymphoid organ development in Lfa-1<sup>d/d</sup> mice**

| Organ          | Wild type | Lfa-1<sup>d/d</sup> | p-value |
|----------------|-----------|---------------------|---------|
| Thymus<sup>a</sup> | 70.4 ± 4.8 | 61.8 ± 4.3 | NS      |
| Bone marrow<sup>b</sup> | 35.0 ± 7.3 | 29.0 ± 4.1 | NS      |
| Spleen<sup>b</sup> | 71.9 ± 7.0 | 95.1 ± 8.4 | 0.040   |
| Lymph nodes<sup>a</sup> | 1.31 ± 0.10 | 0.54 ± 0.09 | <0.0001 |
| Peyer’s patches<sup>a</sup> | 0.39 ± 0.04 | 0.11 ± 0.02 | <0.0001 |
| Blood<sup>c</sup> | 2.2 ± 0.5 | 2.7 ± 0.4 | NS      |

Data are derived from at least six independent mice per group.

<sup>a</sup>Cells/organ.
<sup>b</sup>Cells/femur.
<sup>c</sup>Cells/ml.
of-function mutation on T cell responses directly, we stimulated splenocyte cultures by incubation with the bacterial superantigen, Staphylococcal enterotoxin B (SEB), which activates Vβ8+ T cells of CD4 and CD8 subsets. The dose–response curve depicted in Fig. 5a demonstrates that proliferation of Lfas−/− T cells was attenuated significantly as compared with wild-type cells at all concentrations of SEB tested. Moreover, in allogeneic mixed lymphocyte reactions with irradiated BALB/c splenic stimulator cells, the proliferation of Lfas−/− T cells was reduced significantly as compared with wild-type T cells (Fig. 5b). To analyze further the effects of the Lfas−/− mutation on T cell activation, the induction of CD25 and CD69 expression on SEB-stimulated T cells was studied. The results in Fig. 5c demonstrate significantly reduced expression of CD25 and CD69 on Lfas−/− as compared with wild-type T cells. Together, these results indicate that T cell activation by superantigen-pulsed or allogeneic APCs is impaired markedly by the Lfas−/− mutation.

To distinguish whether reduced activation of Lfas−/− T cells may result from altered interactions with APCs or from T cell intrinsic defects, purified splenic T cells were stimulated with a range of concentrations of plate-bound CD3 antibody. The results in Fig. 5d clearly show that Lfas−/− T cells proliferate as efficiently as do wild-type T cells in response to plate-bound CD3, which suggests that the Lfas−/− mutation does not cause a general defect of T cells to respond to antigen receptor–mediated stimulation.

In additional experiments, potential mechanisms underlying impaired activation of Lfas−/− T cells were investigated. Expression of activated LFA-1 may improve and extend stable contact formation, which may lead to hyperactivation of T cells and accelerated apoptosis. To address this question directly, T cells were stimulated with SEB for various periods and the fraction of apoptotic Vβ8+ T cells was determined by annexin V staining. The results depicted in Fig. 6a show that under the experimental conditions that were used, activation-induced cell death did not occur in wild-type or Lfas−/− T cells. Instead, when compared with unstimulated Vβ8+ T cells, stimulation with SEB reduced the fraction of apoptotic wild-type and Lfas−/− T cells to a similar extent (Fig. 6a). To investigate further the influence of the Lfas−/− mutation on T cell apoptosis after antigen receptor engagement, SEB was injected into hind footpads of mice. The results in Fig. 6b show that deletion of Vβ8+ T cells caused by in vivo SEB administration was similar in wild-type and Lfas−/− mice. Thus, the αL-GFFKR deletion of LFA-1 does not seem to lead to increased T cell apoptosis after antigen receptor stimulation.

Figure 5. Lfas−/− mutation impairs T cell proliferation stimulated by APCs. Splenocytes from wild-type or Lfas−/− mice were stimulated with the indicated concentrations of SEB (a) or irradiated BALB/c splenocytes or autologous cells (b). [3H]-thymidine incorporation was measured 72 h later (n = 6 independent experiments). (c) Splenocytes were incubated with SEB (10 μg/ml) for 16 h. The percentage of CD4 T cells from wild-type and Lfas−/− mice expressing CD25 and CD69 was determined by flow cytometry (n = 4–5 independent experiments). (d) Enriched splenic T cells were stimulated with plate-bound CD3 antibody, and [3H]-thymidine incorporation was measured after 72 h (n = 4 independent experiments). *P < 0.05; #P < 0.01 (Student’s t test).
Lfa-1\textsuperscript{d/d} mutation does not influence signaling through LFA-1

Integrins may function as signaling receptors, and integrin ligation may trigger signaling processes that influence cellular activation and proliferation (1). Therefore, we addressed the question of whether partial activation of LFA-1 through the Lfa-1\textsuperscript{d/d} mutation may result in constitutive signaling activity or enhanced ligand-induced signaling. LFA-1 ligation was shown previously to induce activation of JNK and Erk1/2 MAP kinases (5). As shown in Fig. 6 c, constitutive levels of phosphorylated JNK or Erk1/2 did not differ between Lfa-1\textsuperscript{d/d} and wild-type cells. Exposure of cells to recombinant ICAM-1(D1-2)-Fc induced robust phosphorylation of JNK, which was not influenced by the expression of activated LFA-1\textsuperscript{d/d}. In addition, Lfa-1\textsuperscript{d/d} and wild-type cells exhibited low, but comparable, levels of Erk1/2 phosphorylation following incubation with ICAM-1(D1-2)-Fc. Enforced expression of constitutively active Rap1, which functions as a potent integrin activator, impaired T cell activation by elevating cellular levels of the cyclin-dependent kinase inhibitor, p27\textsuperscript{Kip1} (28). In addition, LFA-1 may influence cellular p27\textsuperscript{Kip1} levels by its capacity to signal through JAB1 (5, 6), which is involved in the control of p27\textsuperscript{Kip1} degradation (29). Therefore, we compared p27\textsuperscript{Kip1} protein levels in nuclear extracts of Lfa-1\textsuperscript{d/d} (d/d) and wild-type mice (+/+), and protein levels of p27\textsuperscript{Kip1} and laminA/C were determined by Western blot analysis.

Lfa-1\textsuperscript{d/d} mutation reduces lytic activity but not generation of cytotoxic T lymphocytes

To investigate the effects of the Lfa-1\textsuperscript{d/d} mutation on T cell functions in more detail, mice were immunized with ovalbumin and CpG-DNA as adjuvant and cytotoxic T cell activity was determined in vitro. These conditions of immunization result in a strong activation of APCs and render the generation of antigen-specific cytotoxic cells independent of CD4 T cells (30, 31). The results in Fig. 7 show that the Lfa-1\textsuperscript{d/d} mutation did not alter p27\textsuperscript{Kip1} levels. Thus, constitutive or ligand-induced signaling through LFA-1 does not seem to be influenced by the Lfa-1\textsuperscript{d/d} mutation.
toxic T cells in Lfa-1d/d mutant mice or a reduced capacity of these cells to kill target cells. To distinguish between these possibilities, antigen-specific CD8 T cells were quantitated with SIINFEKL-loaded H-2Kb tetramers after immunization of mice with ovalbumin and CpG-DNA. As depicted in Fig. 7 b, the proportion of tetramer-binding CD62lo CD8 T cells was greater in Lfa-1d/d mice than in wild-type mice (0.81 ± 0.13% for Lfa-1d/d mice versus 0.34 ± 0.08% for wild-type mice; n = 6; P < 0.01), whereas comparable tetramer staining was observed in the CD62hi subset of CD8 T cells (0.36 ± 0.08% for Lfa-1d/d mice versus 0.53 ± 0.11% for wild-type mice; n = 6; P = 0.394). These results suggest that the Lfa-1d/d mutation impairs the lytic activity, but not the generation, of CD8 T cells.

Impaired humoral immune response in Lfa-1d/d mice

The Lfa-1d/d mutation was found to perturb cell contact-dependent responses of immune cells in vitro. To investigate further the effects of the Lfa-1d/d mutation on immune responses in vivo, the development of T cell–dependent humoral immunity was studied. Mice were immunized with TNP-CGG adsorbed to alum as an adjuvant; TNP-specific antibody production was measured 21 d later. The results in Fig. 8 demonstrate that the production of TNP-specific IgM as well as IgG1 and IgG2a antibodies were impaired in Lfa-1d/d mice; this suggests that T helper 1- and T helper 2-type responses were affected. Therefore, these results indicate that T cell–dependent humoral immune responses are attenuated substantially in Lfa-1d/d mutant mice.

Delayed recruitment of Lfa-1d/d neutrophils in inflammation

LFA-1 is crucial for the recruitment of leukocytes from the blood circulation to inflamed tissues. In vitro experiments have shown that the Lfa-1d/d mutation enhances adhesion to endothelial cells, but impairs transendothelial migration and cell motility on ICAM-1-Fc (Figs. 3 and 4). To elucidate the relevance of the Lfa-1d/d mutation for inflammatory responses in vivo, aseptic peritonitis was induced by thioglycollate injection, and the recruitment of neutrophils (Gr-1hiMac-1hi cells) was monitored. In wild-type mice, i.p. injection of thioglycollate caused a marked and rapid influx of neutrophils (Fig. 9). Peritoneal neutrophil numbers were highest after 6 h and declined thereafter. However, in Lfa-1d/d mutant mice, neutrophil accumulation was delayed substantially. Thus, neutrophil numbers were reduced by >60% in Lfa-1d/d mice as compared with wild-type mice when an-
alyzed 6 h after thioglycollate injection (Fig. 9). At the 12-h time point, comparable numbers of Lfa-1<sup>+</sup>d and wild-type neutrophils were observed in inflamed peritoneal cavities. Therefore, these results show that the Lfa-1<sup>d/d</sup> mutation substantially impairs inflammatory cell recruitment.

**DISCUSSION**

It is well-established that many integrins are preformed as inactive cell surface receptors that are responsive to diverse cellular signals, and result in rapid activation of ligand-binding capacity followed by deactivation of integrins to a resting state (1, 32). Although numerous studies have investigated the molecular mechanisms of integrin activation in vitro, the relevance of dynamic activity regulation for integrin function in vivo remains largely unknown. In the present study, we have addressed this question by the generation and analysis of a mouse strain exhibiting a constitutive germline deletion of the conserved GFFKR sequence in the LFA-1 α<sub>1</sub> subunit. The consequences of this mutation for the LFA-1–mediated adhesion of normal mouse cells were consistent with in vitro studies using cell transfectants (23). Cells from Lfa-1<sup>d/d</sup> mutant mice exhibited an increased constitutive adhesion to purified LFA-1 ligand and endothelial cells and were more responsive to integrin-activating stimuli. However, the maximal adhesive capacity of lymphocytes to ICAM-1 was not influenced by the Lfa-1<sup>d/d</sup> mutation. Importantly, videomicroscopy analyses demonstrated a defective detachment of the trailing edge of Lfa-1<sup>d/d</sup> cells migrating on an ICAM-1-Fc–coated surface, and thereby indicates that the Lfa-1<sup>d/d</sup> mutation impairs de-adhesion of cells from LFA-1 ligands. Consistent with these results, it was shown previously that stabilizing the active form of LFA-1 with mAb24 causes impaired de-adhesion of T cells from ICAM-1 (33). In contrast to the effects on cell adhesion, constitutive or ligand-induced signaling of LFA-1 was not altered in Lfa-1<sup>d/d</sup> cells. Therefore, these results demonstrate that the Lfa-1<sup>d/d</sup> mutation resulted in a constitutive, but partial, activation of LFA-1–mediated cell adhesion that was associated with defective disassembly of LFA-1–mediated cell adhesions.

Lack of LFA-1–mediated cellular interactions in α<sub>1</sub>-deficient mice was shown to cause multiple immune defects (7). Thus, Lfa-1<sup>−/−</sup> mice showed splenomegaly and reduced lymph node sizes, impaired proliferative response of T cells to allogeneic stimulators, attenuated thioglycollate-induced peritonitis, and reduced NK cell cytotoxicity and delayed-type hypersensitivity responses. Therefore, it was conceivable that enhancing interactions of immune cells through constitutive activation of LFA-1 may cause hyperinflammatory responses. However, analysis of Lfa-1<sup>d/d</sup> mice revealed opposite results, and demonstrated that the Lfa-1<sup>d/d</sup> mutation also impaired immune responses. Similar to the phenotype of Lfa-1<sup>−/−</sup> mice, we found Lfa-1<sup>d/d</sup> mice to exhibit enlarged spleens and hypoplastic lymph nodes, to show impaired mixed lymphocyte responses, and to have a reduced recruitment of neutrophils to the inflamed peritoneal cavity. In addition, T cell activation by superantigen-loaded APCs, cytotoxic T cell activity, and T cell–dependent humoral immune responses were impaired markedly by the Lfa-1<sup>d/d</sup> mutation. Therefore, these results suggest that the capacity to deactivate LFA-1 fully may be as important for the generation of normal immune responses as the presence of LFA-1 and the ability to activate its functions.

Cell contacts mediated by LFA-1 are transient; perturbing the kinetics or duration of these cellular interactions may impair recruitment or responses of immune cells (11). The orderly regulation of cell adhesion and de-adhesion is particularly relevant for cell migration (3, 34). Formation of adhesion sites at the leading edge of the cell is required to provide traction sites, whereas cell adhesions have to be disassembled at the rear of the cell to allow it to detach and move forward. Thus, the lack of integrin activation and the inability to deactivate integrins are expected to impair cell migration. In vitro studies using activating antibodies (33) or transfectants expressing LFA-1 mutants (35) have confirmed this hypothesis for lymphoid cells. The results of the present report extend this concept to normal leukocytes and in vivo conditions. We show that lymphocytes from Lfa-1<sup>d/d</sup> mice are impaired severely in their capacity to migrate on ICAM-1–Fc and to translocate through endothelial monolayers in vitro, whereas in vivo, the recruitment of Lfa-1<sup>d/d</sup> neutrophils in a model of aseptic peritonitis was delayed strongly. In addition, the reduced migratory capacity of lymphocytes may contribute to alterations of secondary lymphoid organ cellularity in Lfa-1<sup>d/d</sup> mice. It is conceivable that the hypoplasia of lymph nodes and Peyer’s patches in Lfa-1<sup>d/d</sup> mice results from the partial inability of lymphocytes to cross high endothelial venules, and that cells that are unable to enter these organs accumulate in spleen.

Immune responses involving cell contact–dependent activation and effector function of T cells were impaired markedly by the Lfa-1<sup>d/d</sup> mutation. Thus, T cell proliferation in response to superantigen–loaded and allogeneic APCs, as well as cytotoxic T cell activity, were reduced. These findings are consistent with previous work that showed that stabilizing the active form of LFA-1 by mAb24 inhibited the proliferative response of T cells to antigen and the cytolytic activity of lymphokine–activated killer cells (33). Because the Lfa-1<sup>d/d</sup> mutation and mAb24 treatment disable complete deactivation of LFA-1, impaired resolution of LFA-1–mediated cell adhesions may be a major mechanism that underlies these defective responses. For example, cytotoxic T cells exert their full cytolytic activity by successive interactions with multiple target cells. Therefore, defective de-adhesion of cytotoxic T cells from target cells would reduce the number of target cells encountered and killed. Furthermore, the process of T cell activation by APCs involves successive phases of transient and long-term cellular contacts, followed by the detachment of T cells before they undergo cell division and regain a migratory behavior (36–38). Therefore, defective
de-adhesion of Lfα-1/d T cells may reduce cell proliferation and the subsequent formation of new cell contacts by antigen-activated T cells.

In contrast to the capacity of cytotoxic T cells to lyse target cells, generation of antigen-specific CD8 T cells was not impaired in Lfα-1/d mice using an immunization protocol that renders cytotoxic T cell induction independent of CD4 T cell help (30, 31). A possible explanation for this result may be provided by studies with αo-deficient mice which showed that LFA-1 is not required for the generation of cytotoxic T cells (7). Alternatively, or in addition, CD8 T cells may not be sensitive to delayed de-adhesion from APCs, or activation of APCs by a strong Toll-like receptor agonist, such as CpG-DNA, may dampen the influence of LFA-1 through induction of additional cell adhesion receptors and costimulatory molecules. Moreover, T cells that are activated in local lymph nodes are known to resume their migratory behavior and continue to recirculate after a short stationary period. Therefore, it is also possible that the defect of Lfα-1/d T cells in cell migration impairs the exit of activated Lfα-1/d T cells and, as opposed to wild-type T cells, results in their prolonged accumulation in lymph nodes.

T helper cell–dependent humoral immune responses were impaired substantially in Lfα-1/d mice. Generation of T-dependent antibody responses involves movement of antigen-specific B cells to the edges of B cell follicles where they present antigen to specific CD4 T cells that have migrated to this site after activation by, and detachment from, dendritic cells (39). Activated B cells expand and remain in the follicles to become germinal center cells, or differentiate into plasma cells that migrate to the lymph node medulla and other sites (39, 40). Defective resolution of cell adhesions also may contribute, at least in part, to the reduced T-dependent antibody production in Lfα-1/d mice. This mechanism may attenuate activation and proliferation of antigen-specific T cells, but also may retain these cells in the T cell area, and thereby, prevent interaction with specific B cells. Efficient movement of T and B cells between different anatomic compartments of lymphoid organs may be impaired further by the reduced migratory capacity of Lfα-1/d cells.

To elucidate further the mechanisms underlying impaired immune responses of Lfα-1/d cells and mice, we investigated whether the Lfα-1/d mutation may cause increased apoptosis as a result of hyperactivation of T cells, or alter LFA-1–mediated signaling. However, T cell apoptosis following in vitro SEB stimulation or in vivo administration of SEB did not differ between wild-type and Lfα-1/d T cells. In addition, neither the constitutive nor the ICAM-1–induced activation of Jnk and Erk-1/2 MAP kinases was augmented in Lfα-1/d cells. Finally, the cyclin-dependent kinase inhibitor, p27kip1, that was shown to be up-regulated by overexpression of the integrin activator, Rap1 (28), may be targeted by LFA-1 signaling through JAB1 (29), was expressed at normal levels in Lfα-1/d cells. Activation of purified T cells by plate-bound CD3 antibody also was normal, and thereby confirmed that Lfα-1/d T cells do not exhibit an intrinsic defect to be activated through the antigen receptor.

In summary, we have generated a mutant mouse strain that shows expression of constitutively active integrin LFA-1, and thereby disables complete deactivation of LFA-1. The results demonstrate that deactivation of LFA-1 and resolution of LFA-1–mediated cell adhesion are vital for the generation of normal immune responses in vitro and in vivo. Therefore, the present study supports the concept that dynamic regulation of ligand-binding capacity is a key element of integral function.

MATERIALS AND METHODS
Antibodies and reagents. Antibodies against the surface markers CD3ε (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD11a (2D7), CD11b (M1/70), CD11c (HL3), CD18 (C61/17), CD25 (3C7), CD29 (HMB1-1), CD49b (H4/1), CD49d (R1-2), CD62 (MEL-14), CD69 (H1.2F3), B220 (RA3-6B2), Gr1 (RB6-8C5), T cell receptor Vβ8 (F23.1), and annexin V were from BD Biosciences. Antibodies against p27kip1, laminA/C, (p)ERK, and (p)JNK were from Cell Signaling. Thioglycollate was from Difco Labs and SEB was from Toxin Technology. TNP-CGG and TNP-BSA were from Bioserve Technologies, and alum was obtained from Serva. ConA, PMA, and ionomycin were purchased from Sigma-Aldrich.

Gene targeting and mice. A murine genomic ES-129 BAC library (German Genome Systems, Inc.) was screened by hybridization with a 225-bp murine Lfa-1 cDNA fragment encompassing exon 31 as a probe. BAC fragments were cloned into pBluescript (Stratagene) and fully sequenced. The targeting vector was constructed in pBluescript such that the specific mutation and an additional Hpal–cut were introduced into the short arm. The long arm was flanked by a neomycin resistance cassette and a HSV–thymidine kinase cassette. El4.1 embryonic stem cells were electroporated with the Clal-linearized targeting vector, and the transfected cells were subjected to G418 and gancyclovir selection. Homologous recombined clones were detected by genomic PCR, and confirmed by Southern blot hybridization after digestion of embryonic stem cell DNA with Hpal. Single integration was verified by probing the Southern blot with the neomycin resistance cassette. Geminine transmission of the targeted allele was confirmed by Southern blot analysis and the neogene was removed from germline by crossing mutant mice with the cre deleter strain (41).

The mice used in these experiments were homozygous for the mutation and had lost the neocassette. Wild-type littermates were used as controls. Genotyping for the Lfα-1/d mutation was performed by PCR (forward: 5′-AGGCCCTGGCTATCCTAGACTC-3′; reverse: 5′-GGTCTCCTGAGATGCCAGTTTCTCGG-3′) followed by Hpal digestion of the PCR product. Mice were kept according to national guidelines for animal care in a specific pathogen-free animal facility. Animal studies were approved by the Regierung von Oberbayern.

Adhesion assays. ICAM-1(D1-2)-Fc and VCAM-1-Fc fusion proteins containing the human Fcγ1 fragment were purified from supernatants of transfected 293T cell line using Protein A-Sepharose columns. For cell adhesion assays, 96-well plates were coated with 1.5 μg/ml purified ICAM-1(D1-2)-Fc, VCAM-1-Fc (0.8 μg/well), or Fcγ1 fragments (1.0 μg/ml) overnight at 4°C. The plates were washed with HBSS and blocked with 1% BSA for 1 h at 37°C. Cells were labeled for 30 min at 37°C with 6 μg/ml H33342 dye (Calbiochem) in HBSS and washed twice with HBSS. The cells (2 × 10⁴ cells/ml) were resuspended in HBSS and preincubated with 1 mM MnCl₂, 20 μM g/ml PMA, or a combination of both for 10 min at 37°C. Subsequently, cells were allowed to adhere for 30 min at 37°C, and nonadherent cells were removed by inverse centrifugation for 10 min at 50 g. Adhesion assays were quantified by fluorimetry using a BioLumin 960 (Molecular Dynamics). For all experiments, nontargeted adhesion to Fcγ1 fragments was
subtracted from adhesion to ICAM-1(D1-2)-Fc or VCAM-1-Fc to calculate the fraction of cells adhering specifically to integrin ligands.

To stimulate T cell adhesion through the antigen receptor pathway, lymph node T cells were stimulated with PMA (10 μg/ml) and ionomycin (1 μg/ml) for 2 d. After washing to remove PMA and ionomycin, T cells were maintained in IL-2 (20 ng/ml) for 2 d. T cell adhesion was stimulated by addition of CD3 antibodies (2 μg/ml) for the indicated time periods.

Adhesion assays with bEnd.5 endothelial cells were performed using 16-well glass chamber slides (Nunc). Chamber slides were coated with 2.5 μg/well fibronectin (Roche Diagnostics) and cultured with endothelial cells (2 × 10^5/well) for 2 d. Depending on the experiment, endothelial cells were incubated with 5 μM TNF per well for the last 16 h of culture. After removal of the cytokine, endothelial cells were coincubated with 5 × 10^6 lymphocytes in 100 μl medium supplemented with 10 U/ml rIL-2, which allows expansion of lymphocytes in vivo primed CTL precursors. 51Cr-release assays were performed with EL4 target cells pulsed with 0.1 μM of the OVA-derived peptide, SIINFEKL. Serial dilutions of effector CTL were added to target cells and 51Cr-release was measured after 4 h of coculture. Specific lysis was calculated according to the formula: %specific lysis = (cpm (sample) – cpm (spontaneous release))/cpm (maximal release) – cpm (spontaneous release) × 100. For control, unpulsed EL4 cells were used as targets. Specific lysis of unpulsed EL4 cells was <2% in all experiments.

Ex vivo tetramer staining of CD8 T cells. To investigate generation of cytotoxic T cells, mice were immunized with OVA and CpG-DNA 1668 as described above. 9 d later, draining lymph nodes were analyzed for presence of SIINFEKL tetramer binding, and thus, peptide-specific CD8-positive, CD62L low T cells as described (43). In brief, cells were stained with anti-CD8 APC (clone CD8α; Caltag), anti-CD62L FITC (clone MEL-14), and MHC SIINFEKL tetramer PE (H-2Kk/SIINFEKL [257–264/murine β2-microglobulin streptavidin–PE]) for 1 h at 4°C. Additionally, an Fc block (CD16/CD32, 2.4G2; BD Biosciences) was used to avoid unspecific antibody binding. For life/death cell discrimination, cells were incubated with ethidium monoazide bromid (Molecular Probes).

Thioglycollate-induced peritonitis. Mice were injected i.p. with 0.8 ml thioglycollate (Difco Labs; 40.5 mg/ml). After 0, 6, 12, and 18 h, peritoneal lavage cells were collected and neutrophils were identified by flow cytometry analysis with Gr-1 and Mac-1 antibodies.

Humoral immune responses. Mice were immunized i.p. with 5 μg TNP-CGG (NP19-CGG) adsorbed to alum. Sera were collected before and 21 d after immunization. Total NP-specific immunoglobulin isotypes (IgM, IgG1, and IgG2a) were detected using an ELISA with NP19-CGG–conjugated plates (10 μg/ml in carbonate-buffer, pH 9.5). Sera were diluted 1:500. Murine NP-specific antibodies were detected with alkaline phosphatase–conjugated isotype–specific detection antibodies that were obtained from BD Biosciences (anti-IgG1, anti-IgG2a) or Jackson ImmunoResearch Laboratories (anti-IgM).

Online supplemental materials. Activated wild-type (Video 1) or LFA-1Δ3 (Video 2) splenic T cells were plated on immobilized ICAM-1-Fc (50 μg/ml in the presence of 6 mM MgCl₂). A phase image was taken every 10 s. Thus, 1 s of film equals 1 min real time. Images were taken with a 40× lens and AQM300 Kinetic Acquisition Manager software (Kinetic Imaging Ltd.). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20041850/DC1.

This work was supported by the SFB576, as well as grants HO 1015/6-1 and 6-2 (to B. Holzmann) and PF 259/2-6 (to K. Pfeffer) of the Deutsche Forschungsgemeinschaft, and a grant from the Kommission für Klinische Forschung, Klinikum rechts der Isar (to B. Holzmann) and the Cancer Research UK (to A. Smith and N. Hogg).

The authors have no conflicting financial interests.

Submitted: 7 September 2004
Accepted: 28 April 2005

REFERENCES

1. Hynes, R.O. 2002. Integrins: bidirectional allostERIC signaling machines. Cell. 110:673–687.

2. Bouvard, D., C. Brakebusch, E. Gustafsson, A. Azröd, T. Bengtsson, A. Berna, and R. Fässler. 2001. Functional consequences of integrin gene mutations in mice. Curr. Res. 89:211–223.

3. Hogg, N., M. Laschinger, K. Giles, and A. McDowall. 2003. T-cell integrins: more than just sticking points. J. Cell Sci. 116:4695–4705.

4. Bachmann, M.F., K. McKall-Fairen, R. Schmits, D. Bouchard, J. Beach, D.E. Spener, T.W. Mak, and P.S. Ohashi. 1997. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. Immunity. 7:549–557.
1. Dustin, M.L., T.G. Bivona, and M.R. Philips. 1998. Transendothelial migration and trafficking of leukocytes in LFA-1-deficient mice. Eur. J. Immunol. 28:1959–1969.

2. Berlin-Rufencach, C., F. Otto, M. Mathies, J. Westermann, M.J. Owen, A. Hamann, and N. Hogg. 1999. Lymphocyte migration in lymphocyte function-associated antigen (LFA)-1-deficient mice. J. Exp. Med. 189:1467–1478.

3. Hamann, A., D. Jablonski Westrich, A. Duijvestijn, E.C. Butcher, J.J.L. Simard, G. Duncan, A. Wakeham, A. Sharmaian, A. van der Heiden, M.F. Bachmann, et al. 1996. LFA-1-deficient mice show normal CTL responses to virus but fail to reject immunogenic tumor. J. Exp. Med. 183:1415–1426.

4. Andrew, D.P., J.P. Spellberg, H. Takimoto, A. Villa, L. Rogge, and R. Pardi. 2000. Integrin LFA-1 interacts with the transcriptional co-activator JAB1 to modulate AP-1 activity. Mol. Biol. Cell. 11:1969–1974.

5. Perez, O.D., D. Mitchell, G.C. Jager, S. South, C. Murriel, J. Geginat, A. Villa, L. Rogge, and R. Pardi. 2000. Integrin LFA-1 interacts with the transcriptional co-activator JAB1 to modulate AP-1 activity. Nature. 404:617–621.

6. Schmits, R., T.M. Kündig, D.M. Baker, G. Shumaker, J.J.L. Simard, A. Villa, L. Rogge, and R. Pardi. 2000. Integrin LFA-1 interacts with the transcriptional co-activator JAB1 to modulate AP-1 activity. Mol. Biol. Cell. 11:1969–1974.

7. Takagi, J., and T.A. Springer. 2002. Integrin activation and structural rearrangements as a key regulator of T-cell and antigen-presenting cell interactions and modulates T-cell responses. Nat. Immunol. 3:363–372.

8. Lu, C., J. Takagi, and T.A. Springer. 2001. Association of the membrane tyrosine kinase lyn with CD11b/CD18 molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. J. Cell Biol. 150:545–556.

9. Takagi, K., M. Shimonaka, and T. Kinashi. 2004. Rap1-mediated lymphocyte function-associated antigen-1 activation by the T cell antigen receptor is dependent on phospholipase C-γ1. J. Biol. Chem. 279:11875–11881.

10. Shimonaka, M., K. Katagiri, T. Nakayama, N. Fujita, T. Tsuroo, O. Yoshie, and T. Kinashi. 2003. Rap1 regulates the assembly and adhesiveness of integrin lymphocyte function-associated antigen-1. J. Immunol. 159:268–278.

11. Diamond, M.S., and T.A. Springer. 1993. A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. J. Cell Biol. 120:545–556.

12. Catanzaro, K., M. Hatton, N. Minato, and T. Kinashi. 2004. Rap1 activation through cytohesin-1 and Jun-activating binding protein 1. Nat. Immunol. 4:1083–1092.