LFA-1–deficient Mice Show Normal CTL Responses to Virus but Fail to Reject Immunogenic Tumor

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Summary

The leukocyte integrin LFA-1 (CD11a/CD18) plays an important role in lymphocyte recirculation and homotypic interactions. Leukocytes from mice lacking CD11a displayed defects in in vitro homotypic aggregation, in proliferation in mixed lymphocyte reactions, and in response to mitogen. Mutant mice mounted normal cytotoxic T cell (CTL) responses against systemic LCMV and VSV infections and showed normal ex vivo CTL function. However, LFA-1–deficient mice did not reject immunogenic tumors grafted into footpads and did not demonstrate priming response against tumor-specific antigen. Thus CD11a deficiency causes a selective defect in induction of peripheral immune responses whereas responses to systemic infection are normal.

The CD11a/CD18 (LFA-1) complex, a cell adhesion molecule essential for cell-mediated cytotoxicity by T-lymphocytes (CTL) and natural killer (NK) cells, plays an important role in host defense (1, 2). The natural ligands for LFA-1 are ICAM-1, ICAM-2, and ICAM-3, which have been shown to facilitate interactions between T lymphocytes and endothelial tissues, target cells (3, 4), and antigen-presenting cells (APC) (5, 6). LFA-1 belongs to the β2-integrin family of adhesion molecules, whose members include MAC-1 (CD11b/CD18), p150,95 (CD11c/CD18), and a putative heterodimer recently described (7). The CD11b/CD18 heterodimer has been found to be important for non-specific adhesion of granulocytes and monocytes to endothelial cells, and CD11c/CD18 is reportedly a phagocyte receptor involved in activating cells after binding to bacterial LPS in infected tissues (8). LFA-1 is thought to be more involved in lymphocyte function (9) and is expressed on lymphocytes, monocytes, granulocytes, and bone marrow cells. A hereditary mutation in the β-chain (CD18) of the β2-integrin family results in a reduction or lack of a functional CD11a/CD18 (LFA-1), CD11b/CD18, and CD11c/CD18 heterodimers. Children affected by this lymphocyte adhesion deficiency (LAD) suffer from extreme susceptibility to bacterial and fungal infections (10–13).

Whereas a genetic defect in the CD18 gene results in the obliteration of all three β-2 integrins, CD11a-deficient mice were expected to lack only the LFA-1 family member. Lymphocytes have been shown to require the LFA-1 heterodimer for normal inflammatory responses in vivo (3, 14). Early studies suggested that LFA-1 probably was crucial to early phase induction of immune responses but was not as important for lymphocyte proliferation (1, 15). Other results have implicated LFA-1 in recirculation and effector function of primed T lymphocytes. Simultaneous injection of anti–LFA-1 mAb with 2,4 dinitro-fluorobenzene (DNFB)[1] rechallenge blocked DTH responses in DNFB-primed mice, which accompanied a significant reduction in the number of lymphocytes in lymph nodes draining treated skin (16). In addition, adoptive transfer of LCMV primed lymphocytes treated with anti–LFA-1 mAb blocked the development of DTH responses in naive recipient animals (17).

Early studies with T-cell lines indicated a possible functional role for LFA-1 in CTL killing of target cells (1, 18, 19). More recent reports have indicated that tumor-specific CTL against lymphoma cells require LFA-1 expression for normal killing responses (20), and that virus-induced tumor progression is enhanced in anti–LFA-1 treated mice, report-

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[1]Abbreviations used in this paper: DNFB, 2,4 dinitro-fluorobenzene; DTH, delayed-type hypersensitivity; ES, embryonic stem cells; LCMV, lymphocyte choriomeningitis virus; VSV, vesicular stomatitis virus.

edly due not to defects in induction of virus-specific CTL but, rather, as a result of impaired CTL killer function (21).

The role of LFA-1 in recirculation has been supported by in vitro and in vivo studies. Monoclonal antibody to LFA-1 inhibits in vitro adherence of lymphocytes to lymph node HEV prepared from frozen sections and significantly reduces migration of lymphocytes into lymph nodes and Peyer's patches (22). The presence or absence of ICAM-1 on HEV has no modulatory effect on lymphocyte attachment and rolling at HEV (4). Lymphocytes expressing high levels of MEL-14 are also less suceptible to the effects of anti–LFA-1 antibody blocking effects on lymphocyte adhesion to HEV (22). This data on the role of LFA-1 in T cell recirculation raises questions as to the role of the molecule during normal homing responses in vivo.

Splenocytes deficient for ICAM-1 are almost totally defective as stimulators in an MLR (6). Antibody-blocking studies against ICAMs 1, 2, or 3 showed that dendritic cell APC function for T cells was abrogated by mAbs against ICAM-1 or 2 but not by mAb directed at ICAM-3 (5). Both induction and expansion of lymphocytes has been shown to require LFA-1. Anti–LFA-1 mAb block T cell induction phase to alloantigen but have no effect on proliferation during bulk expansion of alloresponding T cells (23). These studies and others have revealed that the requirement of LFA-1 by T cells for proliferative responses to antigen can vary depending on the type of APC (24). Clarification is needed on the role of LFA-1 in the induction antigen-specific CTL.

We have disrupted the murine CD11a gene in embryonic stem cells (ES) cells and generated a mouse strain possessing targeted disruption of both alleles of the CD11a gene. We observed reduced proliferative responses of enriched T cells in in vitro MLR and after mitogenic stimulation. Unexpectedly, CD11a homozygous mutant mice mounted normal T cell responses to lymphocyte choriomeningitis virus (LCMV) and vesicular stomatitis virus (VSV) infections and exhibited normal ex vivo CTL function. However, mutant mice showed defective peripheral priming against tumor cells expressing a tumor-specific antigen and in addition were unable to clear tumors injected into footpads.

Materials and Methods

Preparation of Targeting Construct. To prepare a gene targeting construct, a murine 129 genomic DNA library (Stratagene Inc., La Jolla, CA) was screened using oligonucleotides corresponding to the 5' end of the murine CD11a cDNA. A single lambda clone containing exons 1 to 6 was isolated. A construct for homologous recombination was prepared by ligation of a KpnI/SalI fragment (containing the pMC1neo polyA neomcin resistance casette) with a 5-kb 5' XbaI/KpnI fragment and a 0.6-kb 3' SalI/XbaI fragment corresponding to the murine CD11a gene. The long arm of this construct contains exon 1 and part of intron 1, and the short arm contains the 3' portion of exon 6 and part of intron 6. The targeting construct replaces exons 2, 3, 4, 5, and part of exon 6 with the neomycin resistance casette. The first six exons correspond to the signal peptide and the amino-terminal

extracellular region of the CD11a gene. A 500-bp HindIII/BamHI restriction fragment immediately upstream of the region included in the mCD11a targeting construct was used as a probe in Southern blot analysis for targeted events.

Generation of ES Cell Line and Mice. The ES cell line E14 K was electroporated with the CD11a construct, which was linearized at the unique NotI site in the BluescriptII polylinker at the 3' end. Individual colonies were picked after 10 d of selection in 500 μg/ml of G418 and analyzed by PCR using a primer contained within the neo casette (5' AAC GCA CGG GTG TTG GGT CGT TTG 3') paired with an outside primer (5' ACC AGT CTC TGC TTC TTC TGC AC 3') located in the murine CD11a gene 3' to the targeting construct. Positive clones identified by PCR were confirmed using Southern blotting and hybridizing with a flanking probe.

Targeted ES cell clones were injected into day 3.5 C57 BL/6J blastocysts and transferred into the uteri of CD1 pseudo-pregnant foster mothers, as previously described (25–27). Chimeric male and female offsprings were bred to C57 BL/6J, and germ-line transmission of the mutation was documented by PCR and by Southern blotting of tail DNA digested with EcoRI and hybridized with the 5' flanking probe. Mice demonstrating germ line transmission of the targeted allele were then bred to obtain homozygous gene targeted offspring and further backcrosses into C57 BL/6J.

Analysis of Circulating Leukocyte Counts in the Gene-targeted Mice. Peripheral blood of anesthetized mice was collected from the retroauricular plexus in EDTA-capillary tubes or by cardiac puncture at the time of death. Whole blood was diluted 1:3 in Hanks BBS and total white blood cell counts determined on a hemocytometer. The absolute numbers of each leukocyte population were calculated by multiplying total white blood cell counts by differentials. Blood smears were prepared and stained with Leukostat (Sigma Chem. Co., St. Louis, MO) to count differentials.

Flow Cytometry of Gene-targeted Mice. Cell suspensions from thymus, spleen, lymph nodes, and bone marrow of 6–12-wk-old mice were prepared free from red blood cells. For two- and three-color flow cytometry, 5×10^5 cells in 100 μl were stained with antibodies directly conjugated with FITC or PE: anti-CD11a (clone M17/4; PharMingen, San Diego, CA), anti-CD4 (clone YTS 191.1.2; Cedarlane Labs. Ltd., Hornby, ON), anti-CD8 (clone YTS 169.4; Cedarlane Labs.), anti CD18 (clone C71/16), anti CD11b/Mac-1 (clone M1/70), anti-CD3 (clone145 2C11), anti-B220 (clone RA3-6B2), or anti-GR1 (clone RB6-8C5; all PharMingen). Cells were incubated at 4°C for 30 min, washed three times, and fixed in PBS/1% paraformaldehyde before analysis on a FACScan® instrument (Becton-Dickinson, Mountain View, CA). FACS® analysis was performed with Lysis II (Becton-Dickinson). Windows for analysis of granulocytes and lymphocytes were set using side scatter and forward scatter.

Contact Hypersensitivity. Contact hypersensitivity was elicited in mice by using DNFB. In brief, 6–8-wk-old mice were immunized on the shaved abdomen with 50 μl 0.5% DNFB in 4:1 acetone/olive oil at day 0 and day 1. At day 5 these mice were challenged with 10 μl 0.2% DNFB in 4:1 acetone/olive oil on the inner and outer side of one ear. Ear thickness was measured 24 h after reexposition and compared with the untreated ear.

Thioglycollate-induced Peritonitis. Aged and sex-matched mice were injected i.p. with 1.5 ml 4% thioglycollate on d 0. At +4, +24, and +72 h mice were injected i.p. with 8 ml of PBS (without Ca^{++} and Mg^{++}, 2 mM EDTA, 50 μm/ml heparin), their abdomens were massaged and 7 ml of lavage fluid was withdrawn. Peritoneal lavage cells were washed 3X in PBS without Ca^{++}

and Mg^{++} and resuspended in 1 ml of same. Total and differential counts were performed after staining with Turk's solution.

In Vitro Proliferation Assay. Lymph node (LN) cells from +/+, +/− and −/− mice were harvested and 10^5 cells were stimulated hamster anti mouse CD3 mAb (clone 145 2C11; PharMingen) (10 μg/ml) in HL-1 media (Hycor Biomedical, Irvine, CA) containing 2% FCS using 96 well round bottom plates. Cells were pulsed overnight with [³H]thymidine after 24, 48, or 72 h. For cross-linking antibody stimulation 96-well flat bottom plates were used and preincubated with rabbit anti–hamster IgG the night before. Cells were pulsed overnight with [³H]thymidine after 48 h. Proliferation was measured by [³H]thymidine incorporation using Matrix 96-Beta-counter.

Mixed Lymphocyte Reaction (MLR). MLR's were performed by mixing spleen cells from C57BL/6J, bm12, BALB/c or B10.BR animals with responder cells from LN cells or enriched T-cells from LNs and spleen of wild-type or mutant hybrid (129Sv × C57BL/6J) animals. Stimulator spleen cells were irradiated with 2000R, washed twice and 1 × 10^5 cells were cocultured with 1 × 10^5 responder lymph node cells or column enriched T cells (R&D Systems, Inc., Minnneapolis, MN). Assays were performed in quadruplicate in 96-well flat-bottom Microtiter plates in HL-1 media (Hycor Biomedical) containing 2% heat inactivated FCS. Cells were pulsed at day 4 with 1 mCi ([³H]thymidine) for 16 h and proliferation measured by [³H]thymidine incorporation using Matrix 96-Beta counter (Camberra Packard).

Natural Killer Cell Function. For induction of NK activity in vivo, mice were injected intraperitoneally with either 100 μg poly(I):poly(C) or saline on days 0 and 1. On day 2 mononuclear spleen cells were isolated by density gradient centrifugation over lympholite M and resuspended at 4 × 10^6 cells per ml in complete media. Mononuclear cells obtained after in vivo induction of NK activity were coincubated with ^{51}Cr-labeled YAC-1 target cells (gift from R. Miller, Ontario Cancer Institute, Toronto, Ontario). Percent specific lysis is given as: % Sp.Lys. = (experimental counts − spontaneous counts)/(total counts − spontaneous counts).

Homotypic Aggregation of Splenocytes. Spleen suspension from +/− and −/− mice were prepared according to standard procedures by straining spleens through wire mesh. Cells were washed twice and resuspended in DMEM, 5% FCS, Glutamine (200 mM) and b2-ME (10^{-6} M). Then 10^3–10^5 cells per well (1 ml) were added to 24-well plates. Cells were incubated for 24 h in the presence of 100 ng/ml PMA. Cell were observed using light microscopy and aggregation was determined as either present or absent.

Viruses. VSV grown in BHK cells was kindly provided by Dr. Lud Prevec (McMaster University, Hamilton, Ontario, Canada). LCMV (Armstrong isolate) grown in BHK cells was originally obtained from Dr. M.B.A. Oldstone (Scripps Clinic and Research Foundation, La Jolla, CA) (28).

Primary Ex Vivo CTL Assay Using Cell Line Targets. Mice were infected with VSV (2 × 10^6 pfu i.v.) or with LCMV (2 × 10^3 pfu i.v.) and spleens were removed after 6 or 8 d, respectively. Spleen cells were coincubated for 5 h with ^{51}Cr-labeled MC57 (H-2^b) and/or EL-4 (H-2^b) target cells, which were infected with VSV or loaded with LCMV-GP peptide (aa33-41, 50 mM, 2 h at 37°C). MC57 cells and EL-4 were infected with VSV for 2 h (15 pfu per cell) before the assay. Specific lysis was calculated as (experimental release − spontaneous release)/(total release − spontaneous release) × 100%.

Primary Ex Vivo CTL Assay Using Macrophage Targets. LCMV-specific CTL were derived as above and ex vivo CTL function was determined using targets derived from −/− mutant mice.

Target cells were obtained by injecting mice with 1 ml thioglycollate at day −5 and harvesting macrophages by peritoneal lavage at day −1. Macrophages were seeded into flat-bottom 96-well plates and incubated overnight with IMDM 10% FCS containing ^{51}Cr sodium chromate (0.2 mCi/ml). Cells were then pulsed with 10^{-5} M LCMV-gp peptide. After 2 h, cells were washed twice and spleen cells were added. 5 h later, 70 ml were pipetted off and radioactivity in the supernatant determined in a γ-counter. Since cell numbers and ^{51}Cr uptake in individual wells varies, total release of ^{51}Cr was determined in each well by lysing cells with 70 μl of HCl (1 M) and counting radioactivity remaining in the well; counts derived from 70 μl supernatant plus remaining counts were taken as 100%.

Assay for Secondary Responses against VSV. Mice were immunized i.v. with VSV (2 × 10^6 p.f.u., Indiana strain). After 6 d spleen cells were restimulated with specific antigen by adding VSV nuclear protein class-1–binding peptide (50 mM) to bulk cultures as previously described (29). After 5 d, in vitro culture were harvested and tested for specific cytotoxicity against VSV-infected EL-4 (H-2^b) target cells. Specific lysis was calculated as above.

Tumor Rejection. Heterozygous and homozygous mutant mice [129JSv (H-2^b) × C57BL/6J (H-2^b)] were anesthetized and 2 × 10^5 MC57 (H-2^b) fibrosarcoma-cells (generated from C57BL/6J) were injected into left hind footpads. Footpad swelling was monitored daily or every second day and measured using a spring-loaded caliper. Footpad swelling is given in percent as compared to uninjected footpad. MC57gp cells were also injected at 2 × 10^5 cells into the left-hind footpad of mice and injected mice were monitored in the same way, or screened for CTL induction.

In Vitro Restimulation of Cytotoxic T Cell Activity after Immunization with Tumor. MC57-GP is a methylcholantren induced fibrosarcoma (originating from C57BL/6 (H-2^b mice) that has been transfected to stably express an LCMV glycoprotein, which contains the immunodominant epitope for CD8+ T cell responses against LCMV infections (described previously in detail [30]). 2 × 10^5 MC57-GP were injected into the left hind footpads of mice, at day 7 spleens were removed and single cell suspensions restimulated in vitro using irradiated (2,000 rad) LCMV-GP peptide-labeled spleen cells. After 5 d cultures were harvested and tested for generation of LCMV-GP–specific CTL activity against EL-4 target cells, which were labeled with LCMV-GP peptide (aa33-41, 50 mM, 2 h at 37°C). EL-4 cells incubated without peptide were used as controls for specificity. Specific lysis was calculated as (experimental release − spontaneous release)/(total release − spontaneous release) × 100%.

Results

Disruption of CD11a Gene and Generation of Homozygous Mutant Mice. Successfully targeted ES cells possessed a 9-kb fragment on Southern blotting compared to the 13-kb wild-type fragment using a probe from the 5′ flanking region of the CD11a gene (Fig. 1, C and D). All confirmed clones were analyzed with the *neo* probe to ensure single integration events. Targeted ES cell clones were injected into C57/BL/6J blastocysts and male chimeric mice were bred to C57Bl/6J mice. Germ line transmission was confirmed by Southern blotting for two of the independently derived ES cell clones. Southern blotting studies from wild-type (+/+), heterozygous (+/−), and homozygous mu-
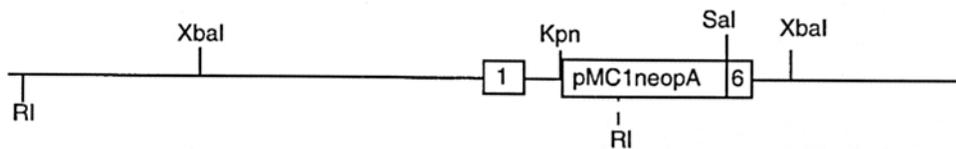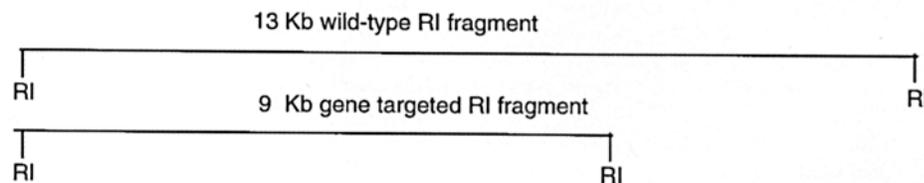
A. Wild-type Allele
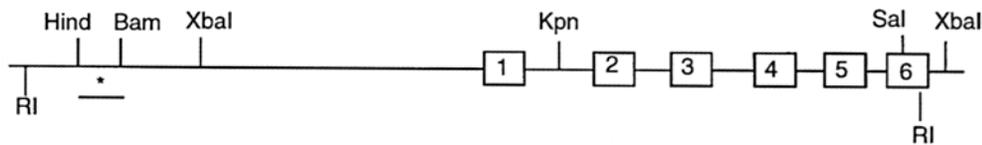
B. Targeting Construct
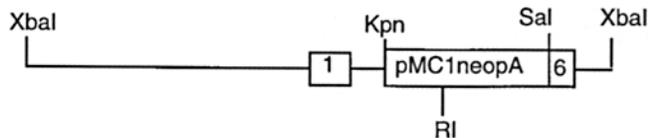
C. Gene Targeted Allele

D. Restriction Fragments

13 Kb wild-type RI fragment

9 Kb gene targeted RI fragment
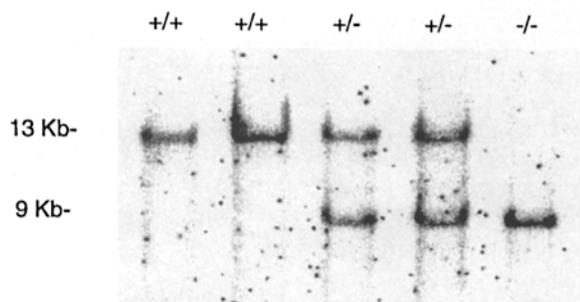
E. Southern Blot

Figure 1

Figure 1. Generation of CD11a deficient mice. (*A*) Restriction map of the murine CD11a. Boxes represent exons 1 to 6; flanking probe is designated *. (*B*) Targeting vector replaces exon 2-5 of the ICAM binding region (44). (*C*) Map of predicted homologous recombination of gene targeting vector. (*D*) EcoR1 digestion and hybridization results in a 9-kb gene targeted fragment compared to a 13-kb wild-type EcoR1 fragment. (*E*) Southern blot of homozygous wild-type and mutant mice. Genotypes are indicated at the top of each lane: +/+ wild-type; +/− heterozygote; and −/− homozygous mutant.

tant (−/−) mice were performed to document the presence of mutation. The expected 13-kb wild-type and 9-kb targeted fragments were observed using the HindIII/BamHI flanking probe (Fig. 1 *E*). Additional digestions and the use of *neo* as a probe confirmed the presence of targeted allele. Heterozygous and homozygous mutant animals were born in expected ratio, survived and grew normally, and were fertile. Animals did not demonstrate any obvious phenotype and remained healthy when maintained under specific pathogen-free conditions.

Splenomegaly and Reduced Lymph Node Size in CD11a −/− Mice. Analysis of 6-wk-old mice (*n* = 6) did not reveal any gross abnormalities in tissue architecture of major organ systems. However, 6-wk-old mutant mice had twofold enlargement of spleen, while lymph nodes were approximately one-fourth normal size, reflecting higher and lower numbers of lymphocytes, respectively (data not shown). An interesting exception of these observations was the finding that mesenteric lymph nodes were of normal size and cellularity.

Lymphocyte Subsets Unaltered in CD11a −/− Mice. Despite differences in size and cellularity of spleen and lymph nodes in mutant mice, surface markers indicated that lymphocyte subset ratios were unchanged in these tissues. Analysis of lymphocyte subsets and peripheral blood leukocytes (PBL) confirmed that B220-, CD3-, and CD11b-positive cells were negative for CD11a expression (Figs. 2 and 3). In addition, the cell surface expression of CD18 was reduced to levels expected for its presence only in the MAC-1 heterodimer (Fig. 3). Since thymocyte selection is influenced by thymocyte avidity with stromal cells, which are known to express ICAM-1 (31), the LFA-1 deficiency might be expected to influence thymocyte ontogeny. However, thymocyte cell numbers were normal (data not shown), as were CD4-CD8 profiles (Fig. 4).

Immunoglobulin Levels Are Normal in CD11a −/− Mice. Ig subclass irregularities can reflect general dysfunction of the immune system (32). Moreover, since LFA-1 and ICAM-1 can be upregulated on T and B cells (3), it is conceivable that CD11a might play a role in T:B cell cooperation and, perhaps, in Ig class switching. We found mutant mice housed under pathogen-free conditions to have normal serum Ig levels (IgM, IgG1, IgG2, IgG2a, IgG2b, IgG3, and IgA), suggesting normal B cell and cognate T:B interactions.
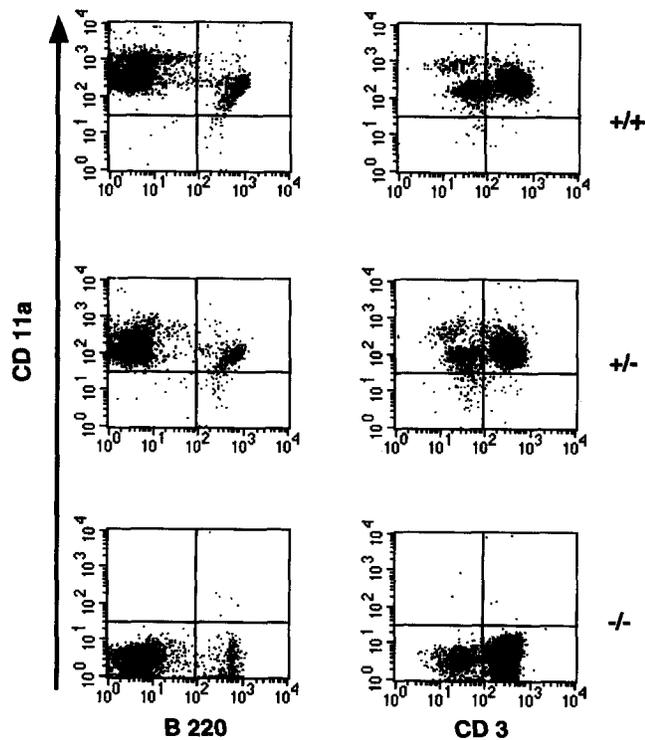
Figure 2. B and T cells lack surface expression of CD11a. Peripheral blood cells were stained with PE-conjugated anti-CD11a and FITC-conjugated anti-B220. Lymph node cells were stained with PE-conjugated anti-CD11a and FITC-conjugated anti-CD3. Mice with homozygous disruptions in CD11a gene show no detectable surface expression. FACS® data was collected for viable lymphocytes according to SSC and FSC.

Homotypic Aggregation Is Defective in CD11a −/− Mice. Peripheral blood lymphocytes, T cells, B cells, and myeloid lineage cells have been shown to aggregate in response to phorbol esters (33–35), and can be blocked by anti–LFA-1 mAb (9). Lymphocytes from LAD patients have also been found not to aggregate in response to phorbol-ester activation (34). To assess leukocyte adhesion in vitro, we incubated spleen cells with PMA for 24 h and monitored for aggregation (33, 36). Leukocytes from heterozygous mice formed aggregates, indicative of homotypic adhesion, whereas leukocytes from mutant mice failed to aggregate in response to PMA (Fig. 5 *A*).

CD11a −/− Enriched T Cells Show Reduced Proliferation. Anti–LFA-1 antibody can inhibit T cell proliferative responses to soluble antigens, alloantigens and mitogens (23, 24). LFA-1 antibodies have been shown to be comitogenic (37). Proliferation assays were used to test T cell responses in MLR. Responder lymph node cells from CD11a −/− mice (H-2^b) showed reduced proliferative responses to BALB/c (H-2^d), B10 BR (H-2^k), and BM12 (H-2^bm12) stimulator cells (Fig. 5 *B*). In vitro lymphocyte proliferative responses to soluble anti–TCR-CD3 antibodies were only 10% compared to controls (Fig. 5 *D*). These results confirm LFA-1 involvement in T cell activation (38). Surprisingly, if CD11a−/− lymphocytes were cross-linked with anti-CD3 antibody fixed to plastic wells, proliferative responses were normal (Fig. 5 *C*), although blast cell formation was delayed by several hours (data not shown).

CD11a −/− Mice Have Reduced Thioglycollate-induced Peritonitis. Peritoneal lavage of thioglycollate-induced peritonitis showed a reduction in the total number of infiltrating leukocytes derived from −/− mutant mice. Neutrophils and monocytes exhibit different time courses for extravasation in response to inflammatory stimuli. Accordingly, neutrophil infiltration at 4 h and monocyte infiltration at 24 h was reduced compared to heterozygous controls (Fig. 6).

CD11a −/− CD8+ T Cells Show Normal CTL Effector Function. Since LFA-1 was initially reported as a molecule centrally involved in CTL and NK cell killer function (1, 2, 18, 39, 40), we naturally investigated CTL function in CD11a −/− mutant mice. Mice were injected i.v. with 2 × 10^3 PFU LCMV at day −8 or 2 × 10^6 PFU VSV at day −6. Spleens from infected mice were removed and splenocytes incubated with ^51Cr-labeled target cells infected with VSV or loaded with LCMV peptide (p33). Contrary to expectations from results of previous reports, splenocytes from CD11a −/− mice were found to mount normal in vivo CTL responses to both VSV (Fig. 7, *A* and *B*) and LCMV (Fig. 7 *D*). CTL from VSV-infected mutant mice lysed both ICAM-1–positive (EL-4) (Fig. 7 *A*) and ICAM-1–negative (MC57) VSV-infected targets (Fig. 7 *B*) (30).

Ex vivo CTL function was also determined using macrophage target cells derived from thioglycollate-injected CD11a −/− mutant mice. This was intended to assess if compensatory changes in CTL from −/− mice might have masked an obvious defect in CTL responses in vivo. We found no significant difference for CTL-mediated lysis us-
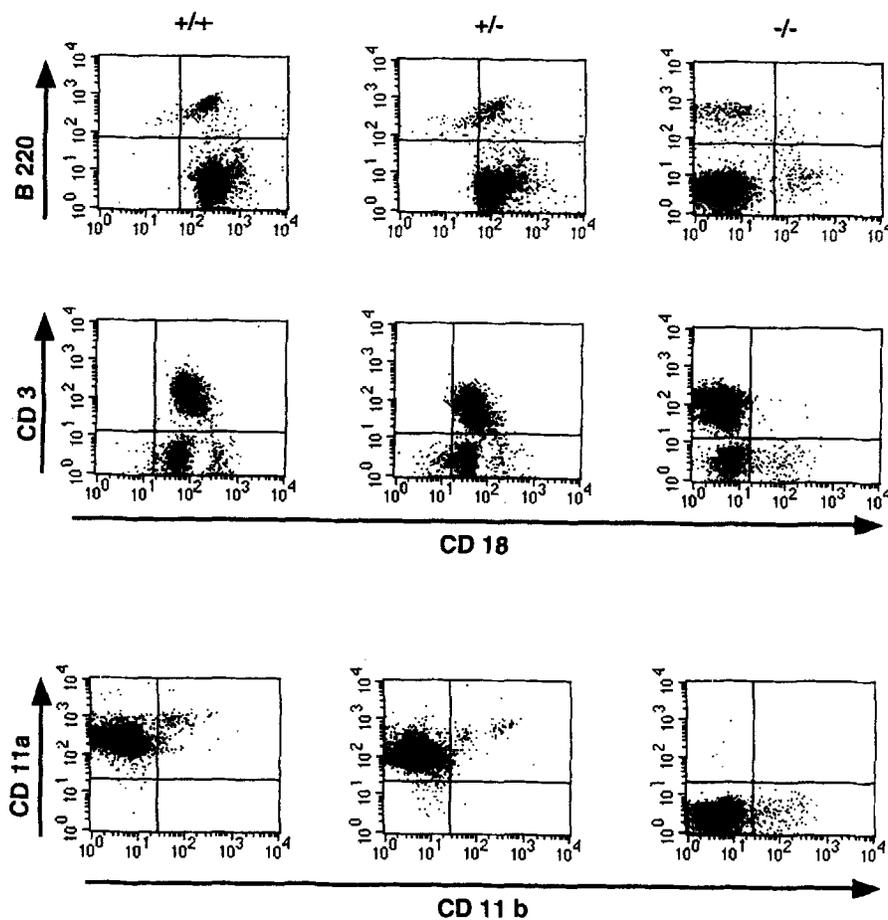
Figure 3. (a) Peripheral blood cells were stained with PE-conjugated anti-CD18 and FITC-conjugated anti-B220 or FITC-conjugated anti-CD3 (top two panels). (b) PBL were stained with PE-conjugated anti-CD11a and FITC-conjugated anti-CD11b. Loss of CD11a expression results in reduction in CD18 surface expression level, which corresponds to expression levels of CD11b on lymphocytes. FACS® data was collected for viable lymphocytes according to SSC and FSC.
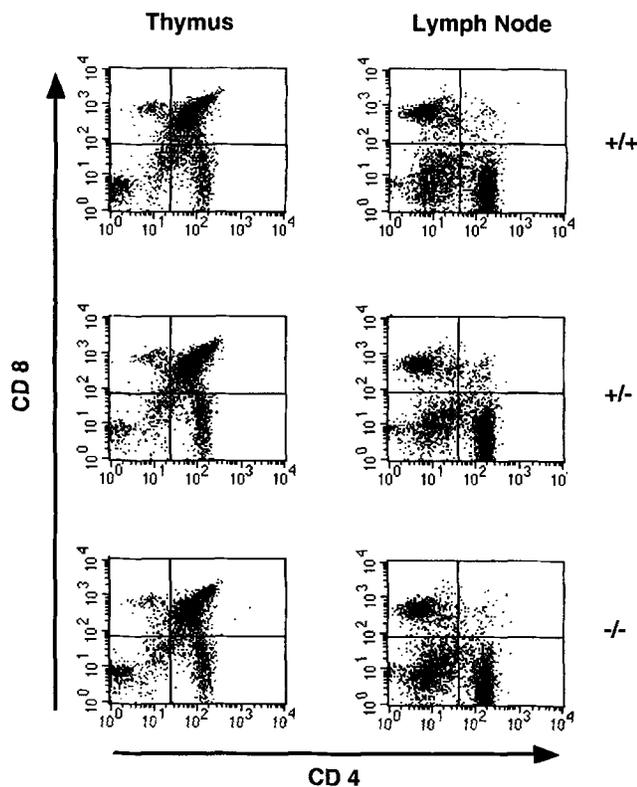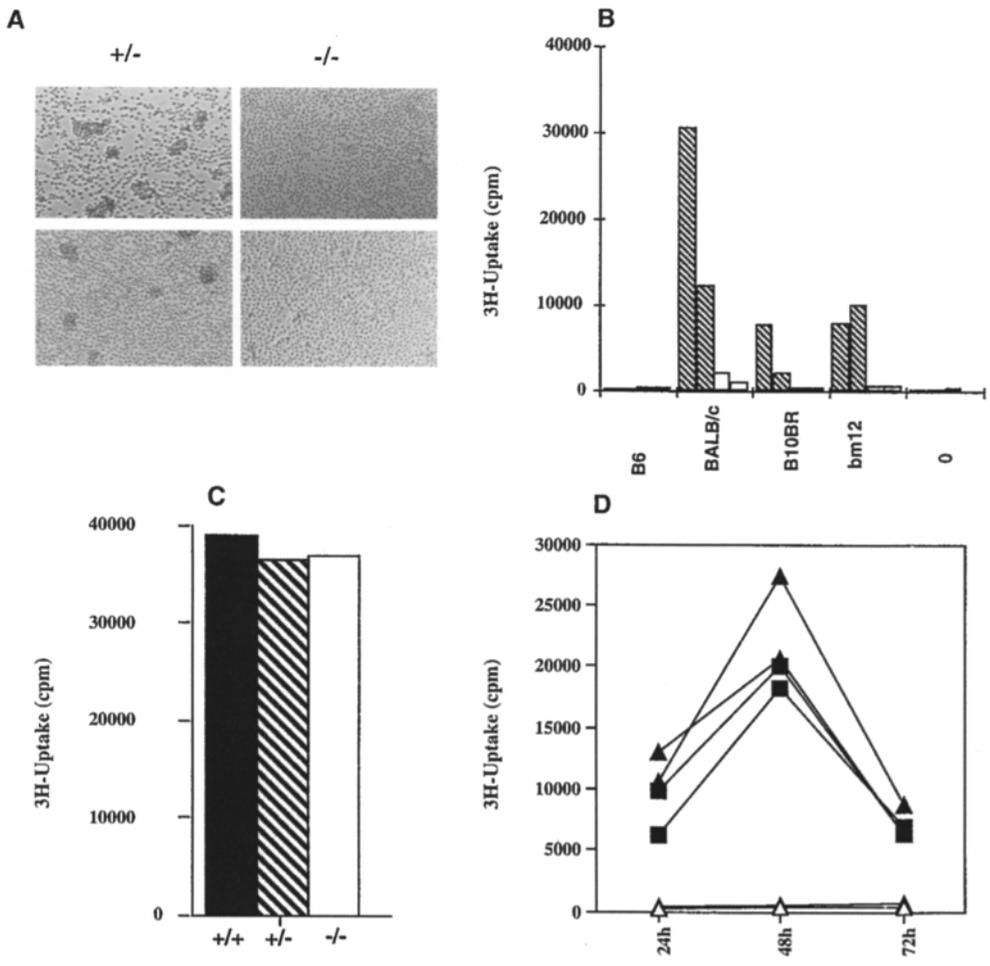
ing +/− or −/− cytotoxic effector cells against −/− targets (Fig. 7, E and F).

Secondary Restimulation Against VSV Is Normal in CD11a −/− Mice. Splenocytes from mice immunized with VSV (2 × 10⁶ PFU) were restimulated with VSV nuclear protein class-1–binding peptide in culture for 5 d. Specific cytotoxicity against VSV-infected EL-4 (H-2ᵇ) target cells was comparable with wild-type control mice (Fig. 7 C). Specific lysis of non-infected EL-4 cells was below 10% for all effector cells. Spontaneous release was below 20% for both targets.

NK Cytotoxicity Is Markedly Reduced. NK cells from CD11a −/− mice were defective in their ability to kill YAC-1 targets (Fig. 8), and this inhibition was comparable to reduced killing observed after treatment with anti-CD11a mAb (41, 42).

Delayed-type Hypersensitivity Is Absent in CD11a −/− Mice. Anti–LFA-1 mAb treatment during either priming

Figure 4. Normal T cell subsets in Thymus and Lymph nodes. Leukocytes from +/+, +/−, and −/− CD11a mice. Thymocytes, lymph node cells, and peripheral blood leukocytes were stained with PE-conjugated anti-CD8 and FITC-conjugated anti-CD4. No detectable difference was seen in T cell subsets from these organs. FACS® data was collected for viable lymphocytes according to SSC and FSC.
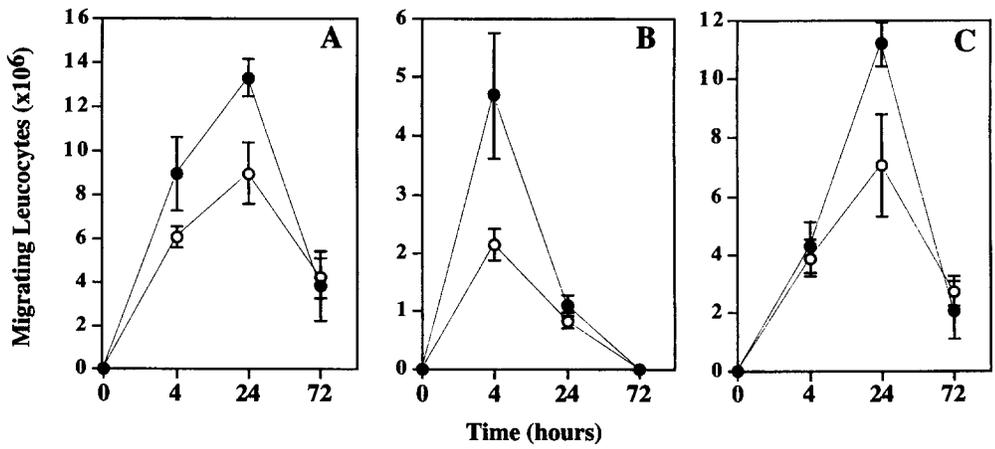
A

+/-   -/-

B

3H-Uptake (cpm)

40000

30000

20000

10000

0

B6   BALB/c   B10BR   bm12   0

C

3H-Uptake (cpm)

40000

30000

20000

10000

0

+/+   +/-   -/-

D

3H-Uptake (cpm)

30000

25000

20000

15000

10000

5000

0

24h   48h   72h

Figure 5. Lymphocyte function in mutant mice. (*A*) Homotypic aggregation of splenocytes from +/−, and −/− mice stimulated with phorbol myristate acetate (PMA 100 ng/ml for 24 h). (*B*) MLR's were performed by mixing 1 × 10⁵ irradiated spleen cells from C57BL/6, BALB/c, B10.BR, or bm12 mice with 1 × 10⁵ lymph node cells harvested from +/− (*crosshatched bars*) or −/− (*open bars*) mice. Each bar represents individual mice. (Results are shown for the 5-d time point; similar results were obtained at 3 and 4 d. Similar results were obtained in three independent experiments). (*C*) Proliferation assay. Lymph node cell suspensions were prepared from +/+ (*black bars*), +/− (*cross-hatched bars*), and −/− (*open bars*) and stimulated. For stimulation by antibody cross-linking, lymph node cells were placed in plates precoated with rabbit anti-hamster IgG and hamster anti-murine CD3 MAb (10 μg/ml). (*D*) Proliferative responses to soluble anti-CD3. 10⁵ lymph node cells were plated into each well and purified hamster anti-murine CD3 added (10 μg/ml). Between 24 and 72 h, proliferating cells were pulsed with [³H]thymidine and cultured overnight. Data represent averages of quadruplicates. (*Closed triangles* +/+; *closed squares* +/−; *open triangles* −/−.)

or rechallenge has been reported to block delayed-type hypersensitivity (DTH) inflammatory responses in mice (16, 17). The DTH responses requires T cell priming in draining lymph nodes and recirculation of T cells to skin. 6–8-wk-old mice were immunized twice with DNFB and rechallenged after 5 d on one ear. After 24 h ear thickness was measured and compared with the untreated ear. Het-

erozygous littermates showed expected visible swelling of the treated ear while DTH was virtually absent in CD11a −/− mice (Fig. 9).

CD11a −/− Mice Do Not Prime/Reject Injected Tumor Cells. We tested the ability of mutant mice to reject a transplant with immunogenic fibrosarcoma tumor cells (MC57, H-2ᵇ). In normal mice, injection of these tumor cells into
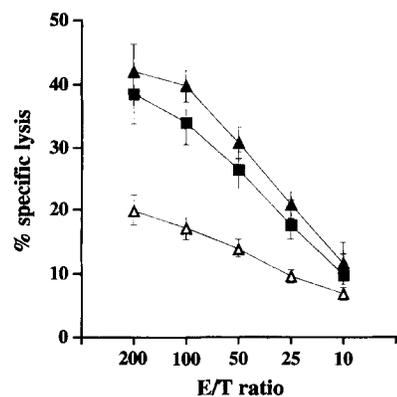

Migrating Leucocytes (×10⁶)

A
16
14
12
10
8
6
4
2
0

B
6
5
4
3
2
1
0

C
12
10
8
6
4
2
0

0   4   24   72

Time (hours)

Figure 6. Leucocyte migration in response to intraperitoneal injection of thioglycollate. Total leucocyte (*A*), polymorphonuclear leucocyte (*B*) and monocyte (*C*) numbers were assessed at the indicated time points after i.p. injection of 1.5 ml of 4% thioglycollate in +/− and −/− mutant mice. Data shown as mean +/− SEM for groups of five age- and sex-matched animals per time point. Solid circles are +/− mice; open circles are −/− mutant mice.

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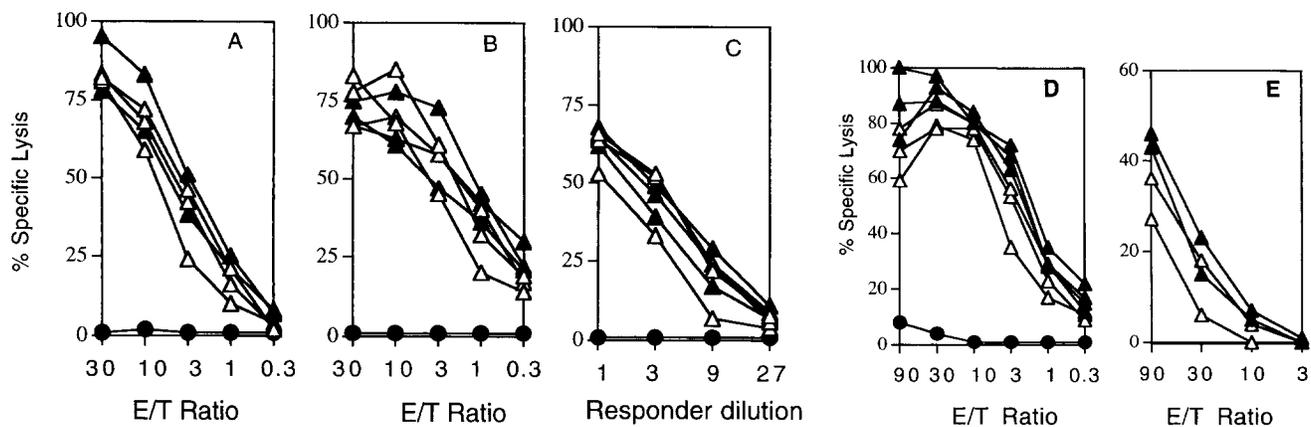
Figure 7. CTL responses are normal in CD11a −/− mice. Normal VSV-specific CTL responses. (*A* and *B*) CD11a +/+ (*closed triangles*) and CD11a −/− (*open triangles*) mice were infected intravenously with VSV (2 × 10^6 PFU). VSV-infected syngeneic targets were either ICAM-1–positive (*A*) (EL-4) or ICAM-1-negative (*B*) (MC57). Closed circles represent non-infected control CD11a +/+ mice. Specific lysis of non-infected control targets <5%; spontaneous lysis of all targets <25%. (*C*) Secondary restimulation against VSV is normal in CD11a−/− mice. Splenocytes from mice immunized with VSV (2 × 10^6 PFU) were restimulated with VSV nuclear protein class-1-binding peptide in culture for 5 d. Specific cytotoxicity against VSV-infected EL-4 (H-2^b) target cells was comparable with wild-type control mice. Specific lysis of non-infected EL-4 cells was below 10% for all effector cells. Spontaneous release was below 20% for both targets. (*D*) Normal LCMV-specific CTL responses. Mice were injected i.v. with 2 × 10^3 PFU. LCMV at day −8 or 2 × 10^6 PFU. VSV at day −6. Splenocytes from infected mice were incubated with ^51Cr-labeled target loaded with LCMV-GP peptide. CD11a −/− mice were found to mount normal in vivo CTL responses. (*E*) Normal ex vivo CTL response to −/− targets. Mice were injected with 2 × 10^3 PFU LCMV at day −8. Macrophages were obtained from −/− mutant mice injected with 1ml thioglycollate at day −5, harvested by peritoneal lavage at day −1 and labeled overnight with LCMV-gp peptide. Targets were incubated with ^51Cr for 2 h before assay. Splenocytes from infected mice were incubated with labeled target cells for 5 h. Open triangles represent −/− effectors; closed triangles represent +/− effectors. (Spontaneous release was below 15% for all assays and ±SD was less than 5% for for each data point.)

footpads induces lymphocytic infiltration of footpads and a visible swelling reaction by 6–9 d, followed by tumor clearance and normalization of footpad thickness within 14 d. CD11a mutant mice showed no swelling at 6–9 d, and developed massive tumor burdens by day 14 (Fig. 10). Histologic analysis of LFA-1 expressing (Fig. 4, *B* and *C*)
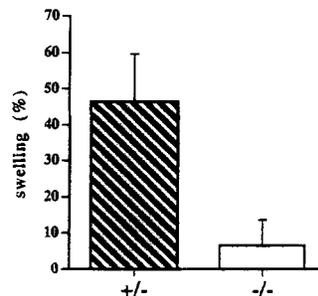
and mutant mouse footpads confirmed the presence of malignant cells and the absence of leukocytes (Fig. 10, *D* and *E*). We then immunized mice into footpads with the fibrosarcoma cell line MC57-gp (the same MC57 cell line as above transfected to express the major immunogenic epitope of the LCMV glycoprotein [30]) to induce CD8^+ CTL responses against LCMV in H-2^b mice (43). 7 d after injection of live MC57-gp into footpads, LCMV-gp–specific CD8^+ CTL became detectable in spleens of CD11a^+/− mice but not in CD11a^−/− mice (Fig. 10 *F*). Accordingly, CD11a^+/− mice, but not CD11a^−/− mice, cleared the MC57-gp tumor cells (data not shown).

Discussion

The role of adhesion molecules during the cellular immune response is multifaceted and involves functions asso-

Figure 8. Natural killer cells are defective in CD11a −/− mice. The NK sensitive cell line YAC-1 was ^51Cr-labeled and used as targets for spleen mononuclear (NK) cells. Percent specific lysis is represented for +/+ (*solid triangles*), +/− (*solid squares*), and −/− mice (*open triangles*). Data represents mean +/− SEM specific lysis for 7 mice of each type analysed in three independent experiments. For induction of NK activity in vivo, mice were injected into peritoneum with 100 µg poly(I):poly(C) on days 0 and 1 and analyzed on day 2.

Figure 9. Absence of DTH response in CD11a −/− mice. Four +/− (*crosshatched bar*) and four −/− (*open bar*) mice were immunized on shaved abdomen and rechallenged on one ear with DNFB. Ear thickness is shown as a percentage of swelling compared to the untreated ear.
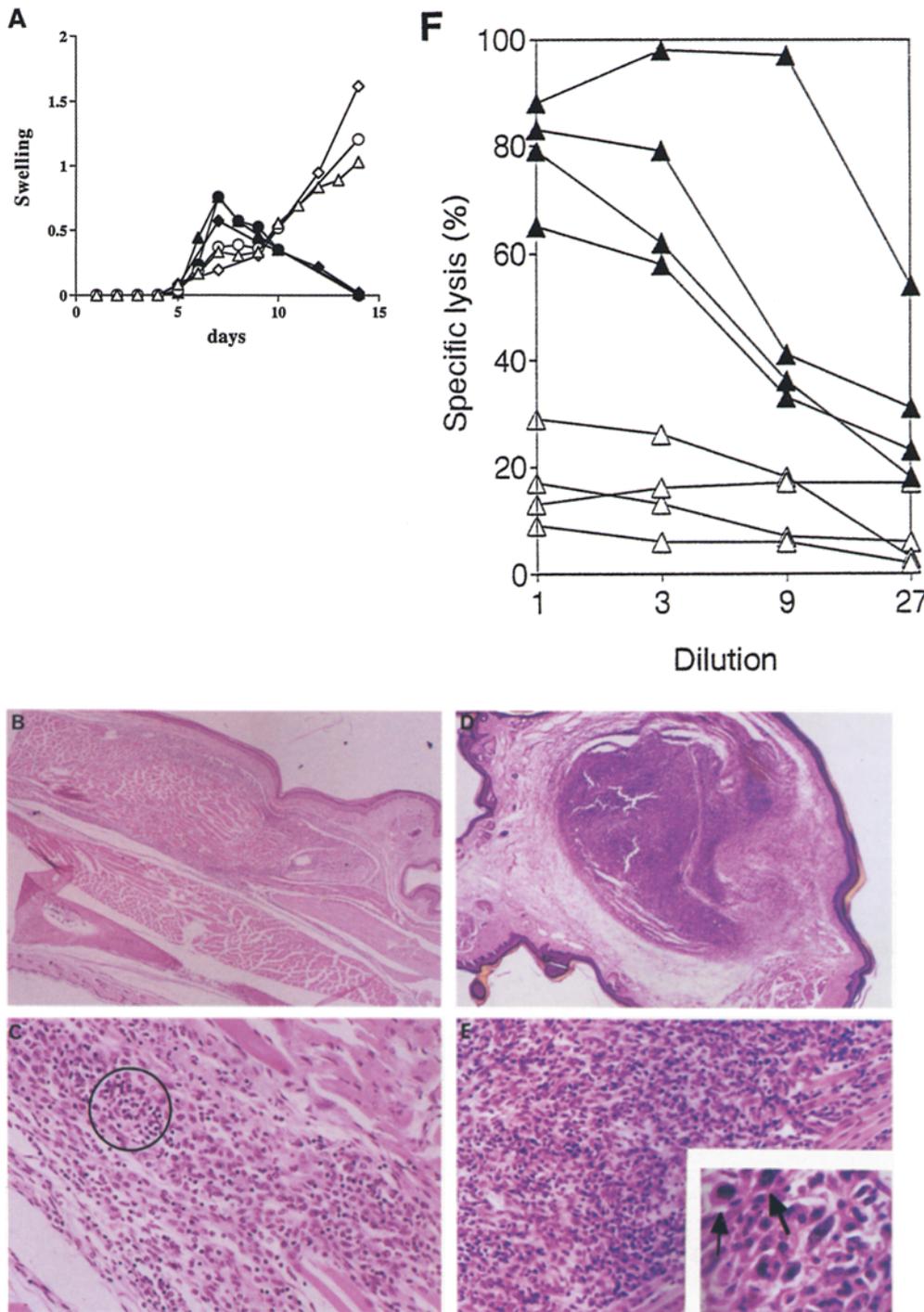
Figure 10. Defective tumor surveillance in mutant mice. (*A*) 2 × 10⁵ MC57 fibrosarcoma cells (C57BL/6 derived) were injected subcutaneously into one footpad of CD11a −/− mice (*open symbols*) and CD11a +/− mice (*closed symbols*). Footpad swelling was monitored daily using a spring loaded caliper. Different markers represent results from three independent experiments, each using 2–4 mice for a total of 10 −/− mutant mice and 10 +/− littermate controls. (*B* and *C*) Histology on footpad from +/− mouse on day 15. Lymphocytes are visible as small dark-stained cells. Circle shows a high concentration of infiltrating lymphocytes. (*D* and *E*) Histology on footpad from −/− mouse on day 15 showing tumor cells. Insert demonstrates mitotic tumor cells (indicated by arrows). (*F*) Day 7 splenocytes from animals injected with MC57-GP (MC57 cell line stably expressing with LCMV glycoprotein) into footpad are restimulated in vitro with peptide for 5 d and assayed for CTL activity using peptide-loaded EL-4 target cells in a ⁵¹Cr release assay. Magnification: *B* and *D*, 150×; *C* and *F*, 750×; *F*, inset, 2250×.

ciated with recirculation through the lymphatic network, interaction with and migration through endothelial lining of blood vessels, cellular adhesion events important during antigen specific activation, and, finally, cell contact during T cell effector functions (reviewed by Springer et al. [9]). In vitro and in vivo findings suggest that LFA-1 is important in lymphocyte migration across the HEV into peripheral LN and Peyer's patches (22). Thus it would be expected that immune reponses to peripheral antigens, where

priming of T cell responses occurrs in the draining lymph nodes, would be defective in LFA-1–deficient mice. We used intravenous infection to assess CTL responses in mutant mice. Because i.v. infection results in a viremia and dissemination of virions lymphocytes are likley to be primed in the spleen, circumventing the need for efficient lymphocyte recirculation through lymph nodes and migration across endothelial barriers.

Nevertheless, in contrast to reports that LFA-1 is crucial

for lymphocyte proliferation against virus-infected cells and CTL effector function (1, 18, 19), ex vivo assays from LCMV- and VSV-infected CD11a −/− mutant mice revealed that LFA-1 deficient CTL had no defect in killing cell line-derived targets. These assays also suggested that antigen-specific CTL had undergone sufficient proliferation in vivo for normal killing responses ex vivo. The implication of these observations is that LFA-1–deficient T cells are capable of undergoing activation and proliferation with no discernable defect compared to normal mice. Reports that lymphocyte proliferation and CTL function is defective after anti–LFA-1 antibody treatment might be a characteristic of the in vitro nature of the assay, or might reflect non-specific effects of antibody treatment, such as capping of other crucial surface molecules or even killing of effector cells. On the other hand, T cells developing in LFA-1–deficient mice may have compensatory alterations in the expression of other molecules that may serve to mask the LFA-1 defect. Ex vivo CTL function using +/− or −/− CTL against targets derived from CD11a −/− mice suggested that CD11a −/− effectors were no better at killing −/− targets than were effector cells that had developed in mice with normal levels of LFA-1. If T cell development in CD11a −/− mice had managed to compensate for LFA-1 deficiency, as evidenced by normal response in vivo to virus, it was not evident in a differential ability to kill −/− targets in vitro. If anything, +/− effectors appeared to be slightly better at killing −/− targets in this sensitive assay, which may reflect a slight outperformance (proliferation and or killer function) of T cells from LFA-1–expressing mice. It should be mentioned that mutant mice also have about twofold greater number of splenocytes than heterozygous mice, thus any minor defect in CTL responses to virus in mutants may be overcome by the sheer excess of T cells present.

Peritoneal lavage after thioglycollate treatment indicated that LFA-1 is not crucial but nevertheless important in extravasation of polymorphonuclear leukocytes and monocytes into the peritoneum in response to inflammatory stimulus. Observed decreases in infiltrate reflected an absolute reduction in cell numbers rather than a shift in the kinetics of the inflammatory response. This finding suggests a significant role for LFA-1 in neutrophil and monocyte responses to local infection.

DTH responses against DNFB were virtually absent in LFA-1–deficient mice. Moreover, immunization in the base of the tail with KLH or OVA in CFA or incomplete FA had little affect on the size of inguinal or paraortic LNs, whereas similar treatment on heterozygous mice resulted in marked lymphoadenopathy (data not shown). Both of these situations could be explained by a recirculation defect, but do not rule out a defect in priming. The lack of tumor rejection after inoculation of MC57 fibrosarcoma cells into footpads and the failure of LCMVgp-transfected MC57 cells to prime ag-specific responses are also consistent with a recirculation defect in CD11a −/− mice.

Although this is the first report that LFA-1 is crucial for priming immune responses against non-virus-induced syngeneic tumors, these results again do not determine if a defect in priming in draining peripheral LN accompanies a lymphocyte recirculation defect. However, our studies with LCMV and VSV infections suggests that priming is efficient in the absence of LFA-1, at least, as it appears, in the context of virus infections in the spleen. Differential priming requirements could reflect the involvement of different types of APC in the peripheral lymph nodes versus the spleen (24). The importance of the strength of the activation signal in LFA-1-deficient cells is exemplified by our finding that proliferative responses of CD11a−/− lymphocytes was absent after treatment with soluble CD3 but relatively normal after treatment with cross-linked anti-CD3 mAb. Our in vitro findings that proliferation of CD11a −/− lymphocytes is significantly reduced contrasts with in vivo responses of CD11a −/− mice to systemic virus infections. Taken together, our results suggest a differential requirement for LFA-1 for lymphocyte activation and proliferation depending, perhaps, on the type of APC involved in presenting antigen to T cells, or, on the microenvironment in which antigen presentation occurs.

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