

β_2 integrins are required for skin homing of primed T cells but not for priming naive T cells

Stephan Grabbe, ... , Cord Sunderkötter, Karin Scharffetter-Kochanek

J Clin Invest. 2002;109(2):183-192. <https://doi.org/10.1172/JCI11703>.

Article

β_2 integrins are of critical importance for leukocyte extravasation through vascular endothelia and for T cell activation. To elucidate the role of β_2 integrins in T cell-mediated immune responses, allergic contact dermatitis (ACD), irritant dermatitis, and delayed-type hypersensitivity (DTH) were assessed in mice lacking the β_2 integrin subunit, CD18. ACD and DTH responses, but not edema formation, were severely suppressed in *CD18^{-/-}* mice. Extravasation of *CD18^{-/-}* T cells into eczematous skin lesions was greatly impaired, whereas migration of Langerhans cell precursors and dendritic cells was normal in *CD18^{-/-}* mice. *CD18^{-/-}* lymph nodes (LNs) contained an abnormal population of CD3⁺CD44^{high} lymphocytes and showed evidence of widespread T cell activation. T cells from regional LNs of sensitized *CD18^{-/-}* mice proliferated in response to hapten challenge, and subcutaneous injection of sensitized syngeneic LN cells directly into ears of hapten-challenged naive recipients restored the defective ACD in *CD18^{-/-}* mice, suggesting that CD18 is not required for priming of naive T cells but is indispensable for T cell extravasation. Thus, a dysfunction of T cells, in addition to granulocytes, may contribute to the pathophysiology of leukocyte adhesion deficiency type I, which arises from mutations in the human *CD18* gene.

Find the latest version:

<https://jci.me/11703/pdf>



β_2 integrins are required for skin homing of primed T cells but not for priming naive T cells

Stephan Grabbe,¹ Georg Varga,¹ Stefan Beissert,¹ Meike Steinert,¹ Gunther Pendl,² Stephan Seeliger,^{1,6} Wilhelm Bloch,³ Thorsten Peters,⁴ Thomas Schwarz,¹ Cord Sunderkötter,^{1,6} and Karin Scharffetter-Kochanek^{4,5}

¹Department of Dermatology, and

²Center for Molecular Biology of Inflammation, University of Münster, Münster, Germany

³Department of Anatomy, and

⁴Department of Dermatology, University of Cologne, Cologne, Germany

⁵Department of Dermatology and Allergology, University of Ulm, Ulm, Germany

⁶Department of Experimental Dermatology, University of Münster, Münster, Germany

Address correspondence to: Stephan Grabbe, Department of Dermatology,

University of Münster, Von-Esmarch-Str. 58, D-48149 Münster, Germany.

Phone: 49-251-835-6536; Fax: 49-251-835-6541; E-mail: grabbe@uni-muenster.de.

Received for publication November 2, 2000, and accepted in revised form December 3, 2001.

β_2 integrins are of critical importance for leukocyte extravasation through vascular endothelia and for T cell activation. To elucidate the role of β_2 integrins in T cell-mediated immune responses, allergic contact dermatitis (ACD), irritant dermatitis, and delayed-type hypersensitivity (DTH) were assessed in mice lacking the β_2 integrin subunit, CD18. ACD and DTH responses, but not edema formation, were severely suppressed in *CD18*^{-/-} mice. Extravasation of *CD18*^{-/-} T cells into eczematous skin lesions was greatly impaired, whereas migration of Langerhans cell precursors and dendritic cells was normal in *CD18*^{-/-} mice. *CD18*^{-/-} lymph nodes (LNs) contained an abnormal population of CD3⁺CD44^{high} lymphocytes and showed evidence of widespread T cell activation. T cells from regional LNs of sensitized *CD18*^{-/-} mice proliferated in response to hapten challenge, and subcutaneous injection of sensitized syngeneic LN cells directly into ears of hapten-challenged naive recipients restored the defective ACD in *CD18*^{-/-} mice, suggesting that CD18 is not required for priming of naive T cells but is indispensable for T cell extravasation. Thus, a dysfunction of T cells, in addition to granulocytes, may contribute to the pathophysiology of leukocyte adhesion deficiency type I, which arises from mutations in the human *CD18* gene.

J. Clin. Invest. 109:183–192 (2002). DOI:10.1172/JCI200211703.

Introduction

β_2 integrins are a group of heterodimeric molecules, including LFA-1 (CD11a–CD18), Mac-1 (CD11b–CD18), and p150,95 (CD11c–CD18), which are involved in leukocyte adhesion and in antigen-specific activation of T lymphocytes (1–3). These cell–cell interactions are generally mediated by binding to intercellular adhesion molecule-1 (ICAM-1) and related molecules. In addition, β_2 integrins serve as multifunctional receptors for a variety of mammalian and microbial glycoproteins, including serum proteins, lectins, and nucleic acids (4–6). At present, four different β_2 integrins have been characterized, all of which are heterodimeric cell-surface molecules consisting of the CD18 molecule and one of the CD11 molecules: CD11a, -b, -c, or -d. CD11a–CD18 is expressed primarily on lymphocytes. CD11b–CD18 is expressed on natural killer cells, monocytes, macrophages, granulocytes, and dendritic cells (DCs). CD11c–CD18 is primarily expressed on DCs, and CD11d–CD18 appears to be expressed on macrophages and lymphocytes (7–9). The human disease known as leukocyte adhesion deficiency type I (LAD I) is characterized by a mutated CD18 molecule, resulting in absent or nonfunctional β_2 integrins. Patients suffering from LAD I exhibit leukocytosis, recur-

rent microbial infections, and defective wound healing (10). We recently generated CD18-deficient mice, which exhibit a phenotype similar to that of LAD I patients and show massive leukocytosis, hyperimmunoglobulinemia, impaired neutrophil extravasation, and spontaneous skin ulcerations. In addition, CD18-deficient mice exhibit defects in formation of aggregates between T cells and antigen-presenting cells (APCs), and in proliferative responses of T cells to alloantigen *in vitro* (11).

Since β_2 integrins are involved in various aspects of innate and antigen-specific adaptive immune responses, we were interested in the role of these molecules in antigen-specific, T cell-mediated immune responses *in vivo*. For this purpose, we investigated the capacity of T cells and DCs to extravasate into inflamed tissue, and used various cutaneous immune responses as experimental models (12). We demonstrate that: (a) T cells from CD18-deficient mice are spontaneously activated *in situ*; (b) CD18-deficient mice are unable to mount allergic contact dermatitis (ACD) and delayed-type hypersensitivity (DTH) responses, but β_2 integrins are not required for priming of naive T cells *in vivo*; (c) extravasation of T cells and granulocytes, but not of mononuclear cells, into sites of cutaneous inflammation is dependent upon

expression of β_2 integrins; and (d) DC migration from blood to normal or inflamed skin, or from skin to regional lymph nodes (LNs), is not mediated by β_2 integrins. Collectively, our data reveal a novel and critical role of β_2 integrins in elicitation of antigen-specific, T cell-mediated immune responses, and suggest that defective T cell extravasation, in addition to inadequate granulocyte or mononuclear cell function, may be responsible for many of the clinical symptoms in patients suffering from LAD I.

Methods

Mice. *CD18^{-/-}* 129 × C57BL/6 (H-2^b) mice were generated as described (11). Mice were used for experiments at 6–12 weeks of age, and were housed according to federal regulations. As wild-type (WT) control mice, we used *CD18^{+/+}* litters from heterozygote crosses. BALB/c, C57BL/6, and C3H/HeN mice, 6–12 weeks old, were obtained from Harlan-Winkelmann GmbH (Borchen, Germany).

Reagents and media. The following haptens and irritants were used: trinitrochlorobenzene (TNCB; Eastman Kodak Co., Rochester, New York, USA), oxazolone (OXA; Sigma Chemical Co., St. Louis, Missouri, USA), dinitrofluorobenzene (DNFB; Sigma Chemical Co.), the water-soluble DNFB analogue 2,4-dinitrobenzenesulfonic acid (DNBS; Sigma Chemical Co.), and croton oil (SERVA Electrophoresis GmbH, Heidelberg, Germany). Mometasone-17-(2-furoate) (0.1%; Essex Pharma GmbH, Munich, Germany) was used to deplete epidermal Langerhans cells (LCs) and Thy-1.2⁺ epidermal T cells as described (13). Approximately 0.2 g of steroid cream per mouse was applied topically to both sides of mouse ears on two consecutive days. For flow cytometry, the following antibodies were used (all obtained from BD Pharmingen, San Diego, California, USA): 145-2C11 (anti-CD3), GK1.5 (anti-CD4), 53-6.7 (anti-CD8), N418 (anti-CD11c), IM7 (anti-CD44), MEL-14 (anti-CD62L), H1.2F3 (anti-CD69), M5/114 (anti-I-A^{b,d,q}, I-E^{d,k}), RB6/8C5 (anti-granulocyte), 30H12 (anti-Thy-1.2), B220 (anti-CD45R), M1/70 (CD11b), 24G2 (Fc block), M1/69 (anti-CD24), RB6/8C5 (Gr-1, anti-granulocytes), and 5H10-1 53-6.7 (anti-CD8). For immunohistochemistry, the monoclonal antibodies ER-HR3 (anti-macrophages), F4/80 (anti-macrophages and dendritic cells), and BM8 (anti-macrophages and dendritic cells) (BMA GmbH, Basel, Switzerland) were used in addition to the above-mentioned antibodies.

ACD, DTH, and irritant dermatitis. ACD experiments were performed as described previously (13, 14). Briefly, *CD18^{-/-}* mice and WT (*CD18^{+/+}*) control mice were sensitized by painting 100 μ l of 0.15% TNCB, or 50 μ l of 2% OXA in acetone/olive oil (4:1) on the shaved abdomen of naive mice. To generate ACD, ears of mice were painted with 10 μ l of 0.8% TNCB or with various doses of OXA (0.5–5%) on both sides of one ear. ACD was determined by the degree of ear swelling of the hapten-exposed ear compared with the vehicle-treated contralateral ear. Ears were measured with a spring-loaded Oditest caliper (Kroepelin GmbH, Schüchtern,

Germany) 24–36 hours after challenge. Mice that were ear challenged without prior sensitization served as negative controls. In some experiments, lymphocytes from inguinal and axillary LNs were harvested 5 days after sensitization, T cells were purified, and 2.5×10^6 syngeneic LN cells or purified T cells (in 25 μ l PBS containing 0.1% mouse serum) were injected subcutaneously into one ear directly before hapten challenge, using a 30-gauge needle.

For induction of irritant dermatitis, 10 μ l of either 0.8% croton oil, 0.5–5% OXA, or of 5% benzalkonium chloride in acetone was painted onto one ear of naive mice. As a measure of irritant dermatitis, ear swelling was determined 16 hours and 24 hours later, using a spring-loaded caliper (14).

For assessment of DTH responses, *CD18^{-/-}* and WT mice were sensitized by subcutaneous injection of 5×10^7 splenocytes from allogeneic BALB/c (H-2^d) donor mice at two sites at the dorsum of naive mice. For elicitation of DTH, mice were challenged by subcutaneous injection of 10^7 BALB/c spleen cells (suspended in 30 μ l PBS) into one hind footpad. Control groups received the same number of spleen cells, but without prior sensitization. DTH was determined by the degree of footpad swelling of the injected site compared with the untreated contralateral footpad; swelling was measured with a spring-loaded caliper 24 or 36 hours after challenge (15).

Immunohistochemistry and immunofluorescence staining of epidermal sheets. Immunohistochemistry was performed as described (16). For preparation of thin sections, tissues were fixed in 4% paraformaldehyde and subsequently treated with 2% osmium tetroxide in 0.1 M PBS for 2 hours at 4°C. They were then washed in 0.1 M PBS, dehydrated in a graded ethanol series, and embedded in araldite. Sections 0.5 μ m thick were stained with methylene blue and examined using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Epidermal sheets were also prepared as described (13). Briefly, mouse ears were mechanically separated into dorsal and ventral sides, and floated on 20 mM EDTA in PBS for 4 hours. Epidermal sheets were collected, washed twice in PBS, and fixed in acetone for 20 minutes at –20°C. They were then washed, and incubated first in 2% BSA for 1 hour at room temperature, then incubated overnight at 4°C in the presence of anti-I-A antibody M5/114 or anti-Thy-1.2 at 1:100 in PBS. The sheets were counterstained with fluorescein-conjugated anti-rat IgG-F(ab)₂ antibody at 1:100 in PBS for 90 minutes at 37°C, washed extensively, and mounted with Aquamount (BDH Laboratory Supplies, Poole, United Kingdom) before visualization with a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Migration of epidermal LCs from skin to regional LNs. Ears were treated with 500 μ g of the fluorescent tracer FITC per 15 μ l dibutylphthalate and acetone (1:1), (supplemented with 5% DMSO; Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). The retroauricular and cervical LNs were prepared 18 hours later.

Single-cell suspensions were stained with phycoerythrin-labeled anti-I-A antibodies, and subjected to flow cytometry.

Generation of DCs and T cells. DCs were generated essentially as described (17, 18). Briefly, single-cell suspensions from murine bone marrow were resuspended in complete medium (RPMI 1640 containing 5% heat-inactivated FCS, 50 μ M β -mercaptoethanol, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 20 μ g/ml gentamicin, all from PAA Laboratories GmbH, Linz, Austria), and seeded into 90-mm tissue culture dishes (Becton Dickinson Biosciences, Heidelberg, Germany) for 2 hours at 37°C in an atmosphere of 10% CO₂. Nonadherent cells were collected and cultured in the presence of 150 U/ml GM-CSF and IL-4 (both from conditioned cell culture supernatants). Culture was performed for 5 days, with a change of complete medium every second day, resulting in generation of cells with the surface phenotype and functional properties of immature DCs. To generate mature DCs, cells were treated as above, but were cultured for 6 days, with additional culture in CD40L for 48 hours, as described (17).

Splenic and peripheral LN T cells were generated as described (17). Cells were purified by passage over a nylon wool column and subsequent immunomagnetic depletion of contaminating cells, using monoclonal antibodies against B220, CD11b, 24G2, RB6/8C5, and CD24 (all obtained from BD Pharmingen). After washing and incubation with goat anti-rat IgG MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), the antibody-negative cell fraction was separated with an automated magnetic cell sorter (autoMACS; Miltenyi Biotec). Purity was controlled by flow cytometry analysis. More than 95% of cells were CD4⁺ or CD8⁺.

Allogeneic mixed lymphocyte reaction and determination of hapten-specific activation of peripheral LN cells. Mixed lymphocyte reactions were performed as described (17). For determination of hapten-specific proliferation, regional LNs were removed 72 hours after sensitization with DNFB (70 μ l of 0.1% and 0.5% DNFB). After pre-purification using nylon wool columns, Thy-1.2⁺ cells were enriched by positive selection using magnetic bead-conjugated antibodies against Thy-1.2 (100 μ l per 10⁸ cells; Miltenyi Biotec), and separation via magnetic cell sorting. Mature DCs were generated from CD18^{+/+} mice, coupled with hapten by incubation in 10 mM DNBS or FITC at pH 7.0 for 10 minutes, and then washed three times to remove unconjugated hapten. Thy-1.2⁺ LN cells from sensitized or control mice (2 \times 10⁵ per well) were plated together with hapten-coupled or control DCs (up to 2 \times 10⁴ per well) in 96-well round-bottom plates in complete medium. After 72 hours of incubation, cells were pulsed with 1 μ Ci ³H-thymidine to assess proliferation.

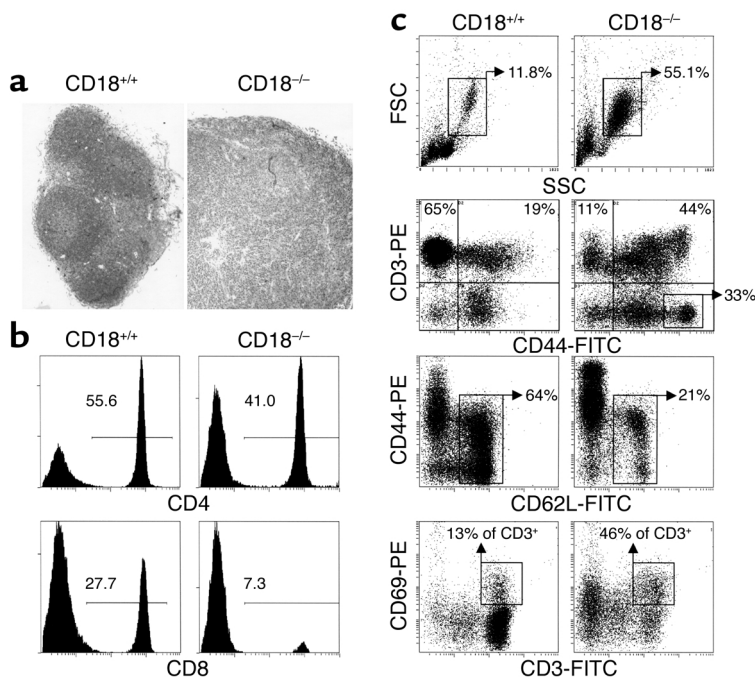
Detection of radiolabeled cells in inflamed skin after intravenous injection. The procedure was performed as described, with modifications (19–21). OXA-sensitized mice were challenged at one ear with 10 μ l 0.5% (wt/vol) OXA, 24 hours before (and in some experiments, also 4

hours before) injection of cells. The untreated ear served as control. Mice were caged individually to prevent allergen contamination of the contralateral ear. Cells for injection were counted, and labeled by incubation at a density of 10⁷ cells/ml medium for 1 hour with 100 μ Ci sodium chromate (containing ⁵¹Cr) (Amersham Pharmacia Biotech GmbH, Freiburg, Germany) per 10⁷ cells. Incubation was done at 37°C and 5% CO₂, in RPMI 1640 and 20% (vol/vol) FCS. After labeling, cells were washed three times in PBS to remove unincorporated ⁵¹Cr. DCs and T cells (5 \times 10⁶ and 1 \times 10⁷ cells per mouse, respectively) were injected into the tail vein of each mouse. After 6 hours, mice were sacrificed and organs were harvested. Ears, lungs, liver, and spleen were counted in a γ counter for 2 minutes per probe (LKB Systems, Ratingen, Germany). The percentage of ⁵¹Cr in inflamed and control ears relative to the sum counted in all organs was calculated.

Results

Lymphatic organs of CD18-deficient mice contain spontaneously activated T cells. As reported earlier (11), and increasing with age, CD18-deficient mice develop profound cervical lymphadenopathy and splenomegaly. Under histopathological examination, the structural organization of the enlarged LNs appeared completely dissolved, with no lymph follicles being visible (Figure 1a). Flow cytometric analysis revealed that the proportion of CD8⁺ T cells was dramatically decreased in these mice (Figure 1b), and a population of large CD3⁺CD44^{high} cells with ultrastructural signs of plasma cells (data not shown) became apparent. Remarkably, the majority of LN T cells showed signs of spontaneous activation (CD3⁺CD44^{high}, CD3⁺CD69⁺, or CD44^{high}CD62L^{low}) (Figure 1c). Similar data were obtained upon investigation of splenic and peripheral blood T cells (data not shown).

CD18-deficient mice are unable to mount ACD and DTH responses. To investigate whether CD18-deficient mice are able to mount T cell-mediated immune responses in vivo, mice were sensitized to one of the haptens, OXA or TNCB. Six days later, contact hypersensitivity responses were elicited, and ear swelling responses were determined. CD18-deficient mice exhibited a profoundly impaired ability to mount a hapten-specific ACD response to either TNCB (not shown) or OXA (Figure 2). This defect could not be overcome by increasing the dose of hapten during sensitization (Figure 2a) or elicitation (Figure 2b) of ACD. Accordingly, the amount of infiltrating lymphocytes and granulocytes was greatly reduced at ACD challenge sites in CD18^{-/-} mice (Table 1 and Figure 3a). Notably, however, a significant number of infiltrating ER-HR3⁺ and I-A⁺ monocytes, macrophages, and DCs were present in ears of hapten-challenged CD18^{-/-} mice (Figure 3b), despite the fact that the ears were not visibly inflamed or swollen. Similar results were obtained using other macrophage and DC markers (Table 1), although the classical markers for macrophages and DCs (CD11b and CD11c, respectively) could not be used for this analysis. This



suggests that immigration of cells of the monocyte/macrophage/DC lineage into inflamed skin is neither secondary to T cell or granulocyte infiltration, nor solely β_2 integrin-mediated.

Similar results were obtained when DTH instead of ACD was investigated. For this, mice were sensitized by subcutaneous injection of 5×10^7 allogeneic spleen cells into the lower back, DTH reactions were elicited by subcutaneous injection of 10^7 allogeneic splenocytes into hind footpads, and footpad swelling responses were determined 24 hours later. *CD18^{-/-}* mice were almost completely unable to mount a DTH response to alloantigen (Figure 4).

In contrast, irritant dermatitis was largely equal in *CD18*-deficient and control mice, since no differences in

the ear swelling response due to epicutaneous application of croton oil (Figure 5a) or to toxic doses of OXA (Figure 2c) were observed. (Only at mildly irritating doses of OXA was a small difference seen.) Upon histopathological investigation, significant tissue edema and vasodilation, but almost no interstitial T cell infiltrate, was found in irritant-exposed skin of *CD18^{-/-}* mice. Whereas many leukocytes remained attached to the inner walls of the dilated blood vessels, some mononuclear cells were present in the interstitial tissue, and appeared to have extravasated from dilated vessels (Figure 5b, arrows).

β_2 integrins are not required for priming of naive T cells in vivo. Since the absence of ACD and DTH responses may be due either to failure in T cell priming or to ineffective transendothelial migration and effector function of

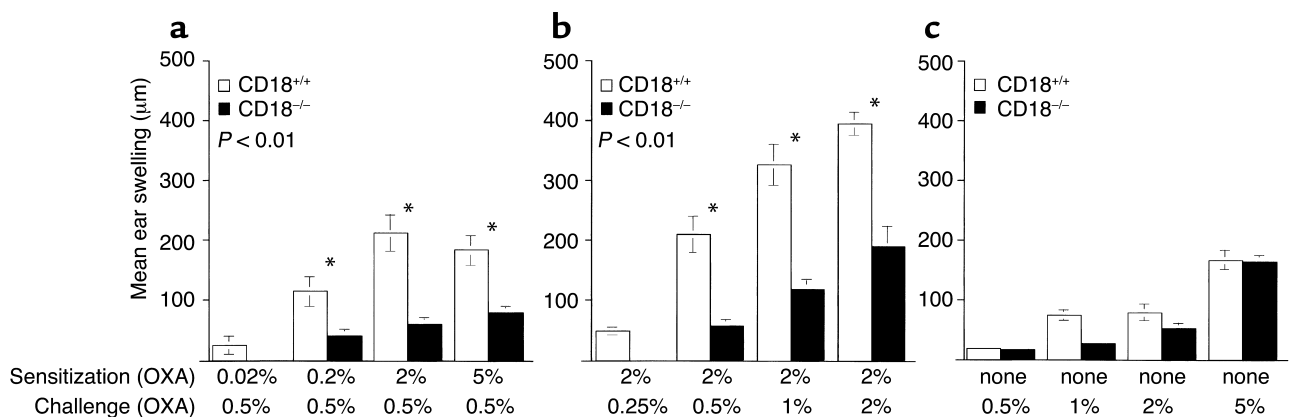


Figure 2
ACD and irritant dermatitis to epicutaneous application of OXA in *CD18^{-/-}* mice. Mice were sensitized to OXA by epicutaneous application of various concentrations of OXA as described in Methods. Six days later, mice were OXA-challenged at one ear to induce ACD, and ear swelling was determined 30 hours later. (a) ACD, titration of sensitization dose. (b) ACD, titration of challenge dose. (c) Irritant dermatitis to epicutaneous application of OXA.

Table 1

Immunohistochemistry of OXA-challenged ears from sensitized *CD18^{-/-}* and WT control mice

Antibody or epitope	Number of cells/hpf	
	<i>CD18^{+/+}</i>	<i>CD18^{-/-}</i>
I-A	30–40	20–25
BM-8	25–30	15–20
F4/80	15–20	5–10
ER-HR3	30–40	15–20
CD4	10–15	1–2
CD8	15–20	1–2
Gr-1	40–50	1–2

Ears were obtained 24 hours after challenge with 0.5% OXA and processed for immunohistochemistry. Ten high-power fields (hpf's) were randomly selected, and the number of antibody-reactive cells were counted in each. Data represent approximate mean numbers of cells per hpf (×400).

primed cells, we tested whether we could detect hapten-specific priming of lymphocytes *in vivo*. As a first indicator of hapten-specific activation, the overall number of LN cells increased to almost the same extent in *CD18^{-/-}* and *CD18^{+/+}* mice (Figure 6a). To investigate whether T cell activation was hapten-specific, regional LN cells were removed 3 days after epicutaneous hapten sensitization, and T cells were isolated and tested for their proliferative response to hapten-pulsed DCs *in vitro*. Although *CD18^{-/-}* T cells proliferated normally in response to stimulation with PMA/ionomycin, mixed lymphocyte reactions revealed that their proliferative capacity after TCR-dependent stimulation was significantly impaired (Figure 6b, inset) (11). Thus, absolute cpm could not be used as a measure of hapten-specific T cell activation. To be able to compare the degree of T cell activation in WT and *CD18*-deficient mice, a stimulation index was calculated between the proliferation due to coculture with hapten-coupled versus uncoupled DCs. As shown in Figure 6b, T cells from DNFB-sensitized mice proliferated upon coculture with DNFB-coupled, but not with FITC-coupled, DCs. Although the proliferative index was somewhat higher in *CD18^{+/+}* mice, especially when mice had been sensitized with low doses of hapten, T cells from *CD18^{-/-}* mice clearly showed hapten-specific proliferation. To further confirm this, we determined T cell activation by flow cytometric analysis of activation-dependent surface molecules. Since most LN T cells had been preactivated in *CD18*-deficient mice, and since we were not using a TCR transgenic system, a profound shift from naive to activated phenotype was difficult to assess. Nevertheless, a dose-dependent increase in the numbers of CD4⁺CD69⁺ and CD8⁺CD69⁺ T cells could be detected, which was similar in extent in both WT and *CD18*-deficient mice (Figure 6c). Thus, T cells from *CD18*-deficient mice were adequately primed *in vivo*.

Defective ACD response in CD18^{-/-} mice is due to the inability of T cells to extravasate into inflamed tissue. Since these experiments revealed impaired proliferative capacity, but no principal defect in hapten-specific priming of

T cells in *CD18^{-/-}* mice, we wished to further elucidate whether defective extravasation of activated leukocytes into sites of ACD challenge accounts for the absence of ACD reactivity in *CD18^{-/-}* mice. To evaluate the relevance of leukocyte extravasation, lymphocyte suspensions were prepared from regional LNs of sensitized mice, and injected intradermally into the ear tissue of naive syngeneic recipients, thereby circumventing the need for leukocyte extravasation. Immediately thereafter, ears were challenged with the relevant hapten at the injection sites, and ACD was determined 24 hours later by measuring ear swelling responses. LN cells from *CD18^{-/-}* mice elicited a significant inflammatory response when injected into hapten-painted ears of naive *CD18^{-/-}* mice (Figure 7, left panel). The intensity of the ear swelling response was similar to that of WT controls, in which naive WT mice were injected with lymphocytes from sensitized WT mice and challenged with hapten. Intradermal injection of purified T cells from sensitized *CD18^{-/-}* mice also restored ACD in syngeneic naive *CD18^{-/-}* mice, and resulted in an ear swelling response that was equal in strength to that of WT control mice treated in an analogous fashion (Figure 7, right panel), although the overall ACD response was somewhat smaller when compared with bulk LN cells (*P* = not significant). These data provide further evidence that T cells were adequately sensitized in *CD18^{-/-}* mice, and also demonstrate that the almost complete absence of an ACD response in *CD18^{-/-}* mice is due to the inability of T cells to extravasate into inflamed skin.

Extravasation of T cells, but not of DCs, is critically dependent upon expression of β₂ integrins. T cells and DCs are the most relevant leukocyte types for elicitation of antigen-specific immune responses. To quantitate their accumulation at inflamed skin sites, T cells and DCs were labeled with ⁵¹Cr and injected intravenously into the tail vein of hapten-sensitized WT mice 24 hours after elicitation of an ACD response at one ear. Immature DCs were used because other studies revealed that immature, but not mature, DCs readily immigrate from blood into inflamed skin (20, 21). Six hours after injection of cells, ears and internal organs were harvested and ⁵¹Cr was counted in a γ counter. As shown in Figure 8, *CD18^{-/-}* T cells had a strongly impaired capacity to home to sites of inflamed skin. In contrast, *CD18^{-/-}* DCs accumulated almost normally at the eczematous ear lesion. Thus, T cells, but not DCs, depend on β₂ integrins for transmigration into inflamed skin.

Since DCs were able to accumulate at sites of ACD, and since mononuclear cells were also detectable in inflamed ears by immunohistochemistry (Figure 3), we investigated whether immigration of resident cutaneous LCs into normal, uninfamed skin was also β₂ integrin-independent. For this purpose, skin sheets from ears of *CD18^{-/-}* mice and WT control mice were stained for I-A⁺ epidermal LCs. As shown in Figure 9a, normal numbers of resident epidermal LCs are present in the epidermis of *CD18^{-/-}* mice. In addition, LCs were

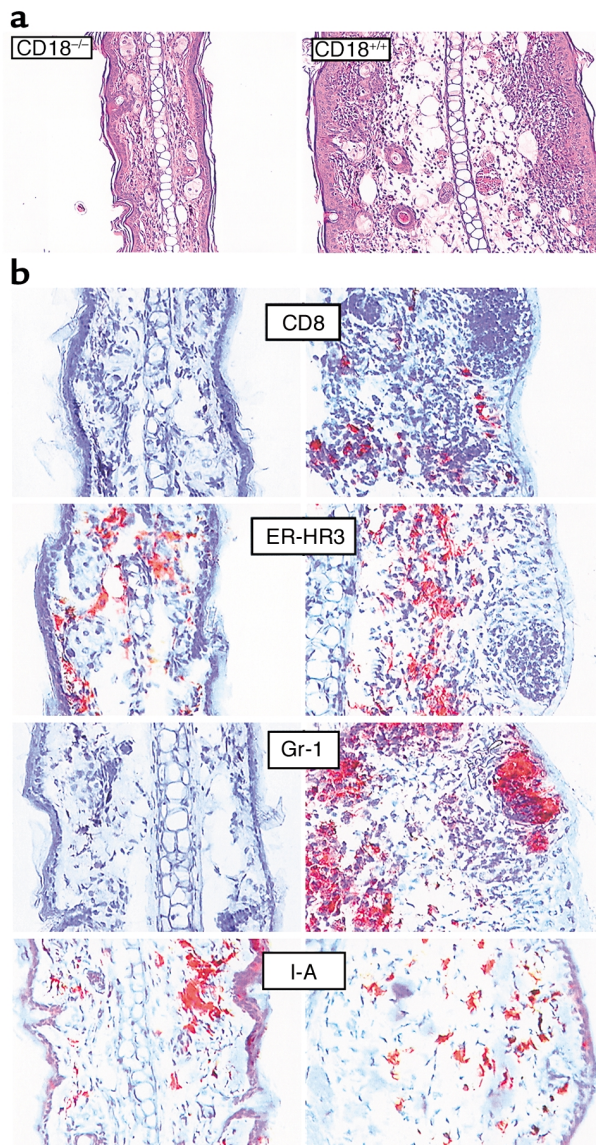


Figure 3

ACD in *CD18*^{-/-} mice. (a) Histology (hematoxylin and eosin staining; original magnification, ×200) and (b) immunohistochemistry (original magnification, ×400) of OXA-challenged ears from sensitized *CD18*^{-/-} and WT control mice, obtained 30 hours after challenge with 0.5% OXA. Antibodies were used to detect the following leukocyte subsets: cytotoxic T cells (detected with anti-CD8), monocytes and macrophages (detected with anti-ER-HR3), granulocytes (detected with anti-Gr-1), and activated macrophages and DCs (detected with anti-I-A).

that β_2 integrin-mediated firm adhesion to endothelial cells is not required for extravasation of DCs into inflamed skin (Figure 3 and Figure 8), or for steady-state immigration of DCs into normal skin (Figure 9a); nor is it required for emigration of LCs from skin to regional LNs (Figure 9b).

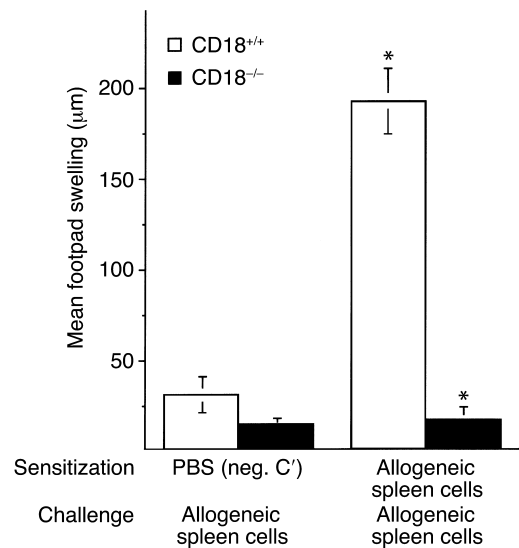
Discussion

β_2 integrins play important roles in T cell activation and in transendothelial migration of leukocytes. Using different models of cutaneous inflammation, we demonstrate that T cells (as well as neutrophils) depend exclusively upon β_2 integrin expression for transmigration into inflamed skin, whereas macrophages and (especially) DCs are both able to migrate into normal and inflamed murine skin without requiring the presence of β_2 integrins. Since significant numbers of monocytes were present in inflamed skin of *CD18*^{-/-} mice, our data suggest that these cells can transmigrate through vascular endothelium using β_2 integrin-independent mechanisms. Likewise, Shang and Issekutz reported that β_1 and β_2 integrins can both mediate monocyte transmigration through a monolayer of synovial fibroblasts (22–24). Thus, in our experiments, the β_1 integrins VLA-4 and VLA-5, interacting with vascular cell adhesion molecule-1, appear to be able to fully compensate for the lack of functional β_2 integrins on monocytes. In contrast, as reported earlier, neutrophils depend entirely upon β_2 integrins for

depleted from the epidermis by topical steroid application, and repopulation of the epidermis with LCs was investigated (13). In WT control mice as well as in *CD18*^{-/-} mice, LCs reappeared in the skin within 2–4 weeks, with the expected kinetics (Figure 9a). Finally, emigration of LCs from the skin toward regional LNs was investigated by epicutaneous administration of the fluorescent hapten FITC, and subsequent assessment of FITC⁺I-A⁺ cells in regional LNs. In both *CD18*^{+/+} and *CD18*^{-/-} mice, we observed a similar increase in the number of FITC⁺I-A⁺ LN cells after epicutaneous application of FITC. Collectively, these observations indicate

Figure 4

DTH in *CD18*^{-/-} mice. Alloantigen-specific DTH was elicited by subcutaneous injection of 10⁸ spleen cells from BALB/c (H-2^d) mice into footpads of allosensitized *CD18*^{-/-} or control (H-2^b) mice, and footpad swelling responses were determined 24 hours later. **P* < 0.001. Neg. C', negative control.



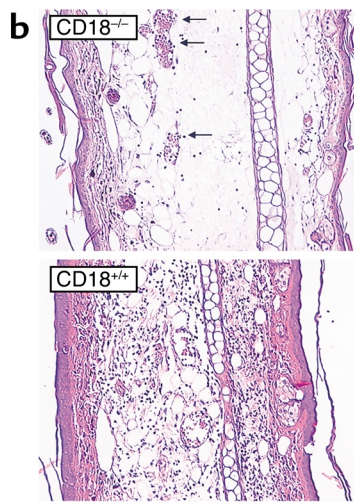
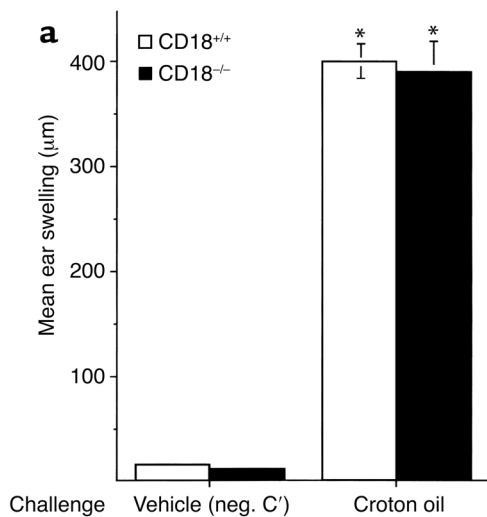


Figure 5

Irritant dermatitis in *CD18^{-/-}* mice. Irritant dermatitis was elicited by application of 10 µl 0.8% croton oil to both sides of one ear, and measurement of ear swelling responses (a) and histological examination (b) were performed 18 hours later. Arrows indicate dilated blood vessels with endothelium-associated leukocytes, some of which appear to have extravasated. Original magnification, ×200. **P* = not significant.

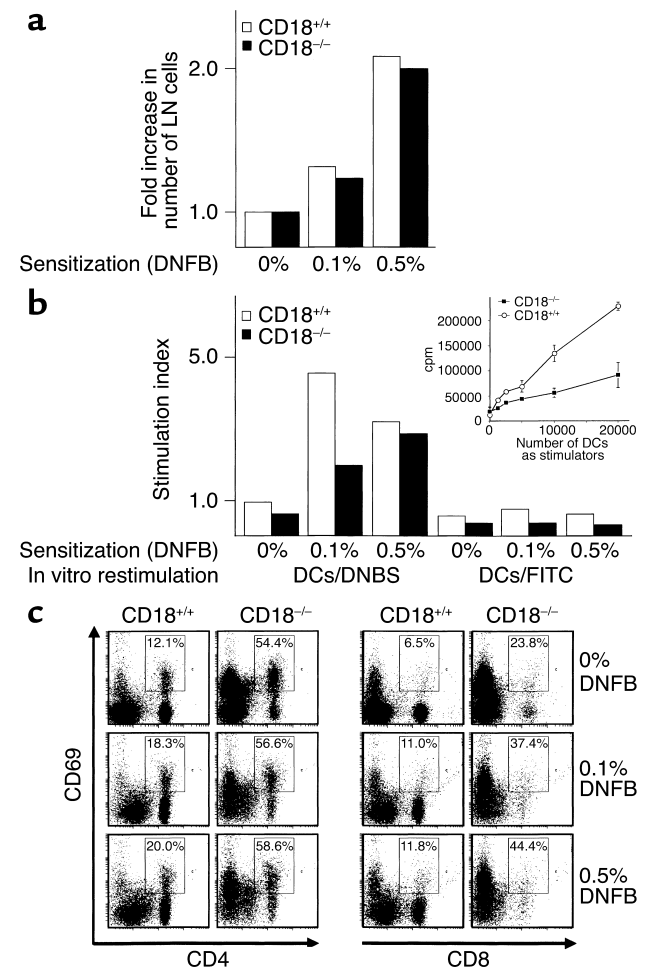
extravasation, since *CD18^{-/-}* mice exhibit an almost complete absence of neutrophil infiltrates (11, 25).

We also demonstrate here that T cell infiltration into acutely inflamed skin is grossly abnormal in *CD18*-deficient mice. This observation correlates with earlier data, obtained by intravital microscopy, showing that leukocyte rolling on endothelial walls in vivo is almost completely absent in *CD18*-deficient mice (11). Similar to our observations, Andrew et al. (26) also found that T cells and neutrophils from LFA-1-deficient mice are unable to transmigrate through endothelial cell layers in vitro, suggesting that LFA-1 is the principal β₂ integrin that mediates transmigration of neutrophils and T cells. These investigators also found that monocytes from LFA-1-deficient mice were able to transmigrate (26), but they suggested that this can be abolished by additional treatment with anti-CD18 or anti-CD11b, which would conflict with the unimpaired monocyte transmigration in *CD18^{-/-}* mice that we observed. In other studies using blocking antibodies against LFA-1, Mac-1, and VLA-4, it was demonstrated that rat monocytes use all three of

these receptors to transmigrate through activated endothelia in vivo, since a blockade of any one of these molecules failed to significantly inhibit monocyte transmigration (22–24, 27). Thus, monocytes apparently are able to vary the type of integrin they primarily use to migrate through inflamed endothelia, depending upon the inflammatory stimulus that activates endothelial

Figure 6

Hapten-specific activation of regional LN cells. Regional LNs were obtained from DNFB-sensitized mice 3 days after sensitization. (a) Fold increase in LN cells after sensitization with either vehicle only (0% DNFB), 0.1%, or 0.5% DNFB. (b) Hapten-specific proliferation of LN cells in response to either the specific hapten, DNBS, or a control hapten, FITC. Thy-1.2⁺ T cells were generated from LN cells and cultured together with hapten-coupled DCs for 72 hours, and then pulsed with ³H for an additional 18 hours. Due to the different proliferative capacities of *CD18^{-/-}* and *CD18^{+/+}* T cells, a proliferation index was calculated as cpm after stimulation with hapten-exposed DCs, divided by cpm after stimulation with untreated DCs. A proliferation index greater than 1 indicates hapten-specific T cell proliferation. Inset: Allogeneic mixed lymphocyte reaction with purified T cells from *CD18^{-/-}* or *CD18^{+/+}* mice as responders, and various numbers of allogeneic DCs (156–20,000) as stimulators. (c) Hapten-specific T cell activation as measured by upregulation of the activation marker CD69. Numbers in boxes indicate the percentage of CD69-expressing cells among the CD4⁺ and CD8⁺ T cells.



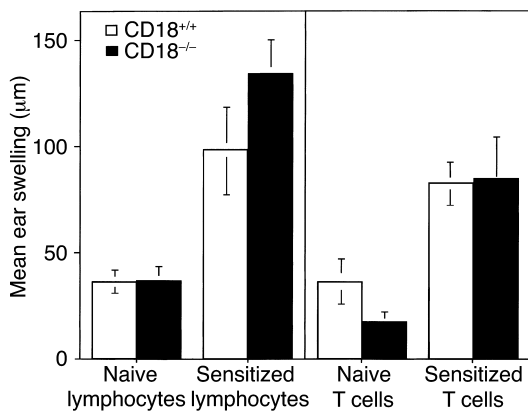


Figure 7
ACD in *CD18*^{-/-} mice after subcutaneous injection of syngeneic LN cells. Left panel: *CD18*^{-/-} and WT mice were OXA-sensitized, and regional LNs were obtained. LN cells (2.5×10^6) from sensitized or naive *CD18*^{-/-} mice were injected into ears of autologous OXA-challenged *CD18*^{-/-} mice, and 2.5×10^6 LN cells from sensitized or naive WT mice were injected into ears of autologous OXA-challenged WT mice. Ear swelling responses were determined after 32 hours. $P < 0.05$ compared with transfer of naive LN cells of the same mouse strain. Right panel: The experiment was performed in a similar fashion as in the left panel, except that purified T cells from *CD18*^{-/-} mice were transferred in all cases. $P < 0.05$ compared with transfer of naive LN cells of the same mouse strain. No significant differences in ear swelling were present between ears injected with whole LN cells or purified T cells.

cells (27). Surprisingly, accumulation of DCs in the uninflamed lung appears to also partially depend on CD18 expression, since decreased numbers of DCs are present in the lungs of *CD18*^{-/-} mice (28). Likewise, intravenously injected, radiolabeled CD18-deficient DCs home less efficiently to the lungs than WT DC (data not shown). In contrast, no striking differences in DC homing were found in normal or inflamed skin (Figure 7 and Figure 8). Thus, extravasation of leukocytes into various anatomical sites may be regulated differentially. This view is supported by findings that emigration of neutrophils into subcutaneous tissue is strongly dependent on expression of CD18, whereas extravasation into peritoneal or alveolar sites is less CD18-dependent (25). Moreover, immigration of rat T cells into cutaneous sites of inflammation is at least partially LFA-1-dependent, whereas infiltration into arthritic joints is independent of LFA-1 or Mac-1 (29–31). Surprisingly, however, DTH responses to sheep red blood cells were normal in LFA-1-deficient mice (32), which contrasts with our findings in *CD18*^{-/-} mice (Figure 4). In another LFA-1-deficient mouse strain (33), ACD responses were found to be virtually absent, which is in complete agreement with our data. These differences may be due to the specific antigen used, or to differences in the experimental systems. In aggregate, these rodent models demonstrate that T cells and neutrophils depend primarily on β_2 integrins to transmigrate into inflamed tissues, whereas monocytes can use mechanisms mediated by LFA-1, Mac-1, and other mechanisms that do not rely on β_2 integrins, for extravasation.

In addition to defects in leukocyte trafficking, CD18-deficient mice display multiple phenotypic and functional abnormalities in various leukocyte subsets. As shown earlier, *CD18*^{-/-} mice develop splenomegaly, cervical lymphadenopathy, and massive leukocytosis, and have serum immunoglobulin levels that are elevated more than 20-fold. So far, investigation of macrophage and APC function has revealed only subtle defects in macrophage function and antigen presentation by DCs (ref. 11 and G. Varga, unpublished observations). Thus, it is difficult to attribute defects in complex immune reactions in vivo, such as ACD and DTH, to a single pathophysiological mechanism. Nevertheless, our data show that the inability of T cells to transmigrate, and not altered T cell activation or function of macrophages or APCs, is the principal defect that abolishes elicitation of T cell-mediated cutaneous immune responses.

The complete inability of *CD18*^{-/-} mice to mount ACD responses is remarkable, since several adhesion and costimulatory molecules, including CD44, VLA-4, ICAM-1, B7-1, and B7-2, have been shown to be involved in elicitation of ACD as well. Antibody blocking experiments revealed that most of these molecules contribute partially to the ACD response, since complete inhibition of ACD could not be achieved with antibodies against any one of these molecules (reviewed in ref. 12). Thus, a cascade of events appears to be required for elicitation of T cell-mediated immune reactions such as ACD. Whereas other elements of this response are dispensable, the presence of CD18-dependent surface molecules on T cells seems to be a *conditio sine qua non* for elicitation of ACD responses.

In addition to leukocyte trafficking, β_2 integrins are of central importance to T cell activation during antigen presentation. LFA-1 is one of the core components of the immunological synapse between APCs and T cells (34),

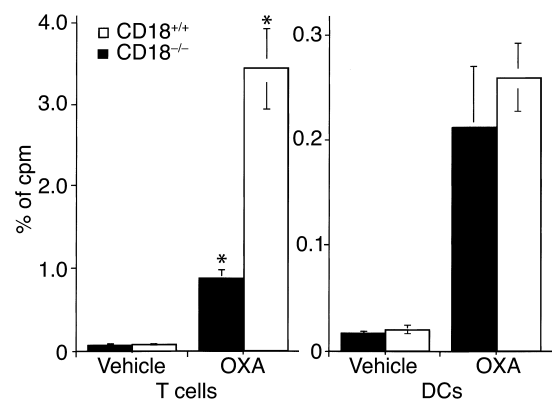


Figure 8
Accumulation of ⁵¹Cr-labeled T cells and DCs at skin sites of ACD. Mice were sensitized and challenged with the hapten OXA. T cells and DCs were generated as described in Methods, labeled with ⁵¹Cr, and injected intravenously 24 hours after hapten challenge. Six hours later, ears and internal organs were harvested and ⁵¹Cr was counted. Data represent percentage of incorporated ⁵¹Cr in the ear in relation to the combined radioactivity uptake in lungs, liver, and spleen. * $P < 0.01$ for WT versus *CD18*^{-/-} T cells; $P =$ not significant for all other groups.

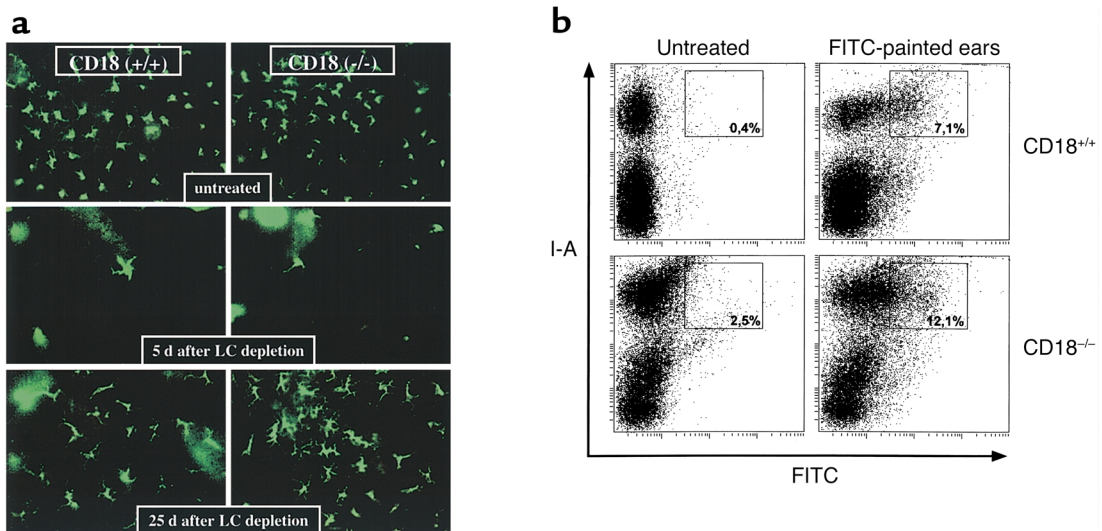


Figure 9

LCs in WT and *CD18*^{-/-} mice. (a) Mice that were either left untreated, or were treated with topical mometasone-17-(2-furoate) 5 days or 25 days prior to assessment of LC density. Ear skin was obtained, and epidermal sheets were prepared and stained for I-A⁺ epidermal cells, as described in Methods. (b) Ears were treated with the fluorescent hapten FITC. Regional LNs were prepared 18 hours later, and LN cells were stained with phycoerythrin-labeled anti-I-A antibodies and subjected to flow cytometry. LCs were gated based on FSC/SSC characteristics. Numbers in boxes indicate the percentage of double positive cells.

and is regarded as a costimulatory molecule by many immunologists. Thus, our earlier finding that T cell proliferative responses to alloantigen are absent in *CD18*-deficient mice, when using a standard mixed lymphocyte reaction (11), was not unexpected. However, when activated mature allogeneic DCs were used as APCs, *CD18*^{-/-} T cells did exhibit alloantigen-specific proliferation, albeit to a significantly impaired degree (Figure 6, inset). Therefore, our earlier observation can be explained by suboptimal antigen presentation when allogeneic splenocytes instead of mature DCs were used as APCs. Likewise, and in agreement with data from work using *LFA-1*^{-/-} mice (32), proliferation of T cells in response to most mitogens or lectins is normal or even enhanced in *CD18*^{-/-} mice (data not shown). In addition to these *in vitro* data, hapten-triggered T cell proliferative responses are only slightly impaired after *in vivo* sensitization, and normal ACD can be induced when the requirement for extravasation of T cells is circumvented (Figure 6 and Figure 7), suggesting that β_2 integrins are not absolutely required for adequate T cell priming and effector function. Quite unexpected was the observation reported here that numbers of activated and/or memory type T cells are greatly increased in *CD18*-deficient mice *in vivo*. This shows that, *in vivo*, LN T cells in *CD18*-deficient mice are not activation-deficient, but are diffusely hyperactivated compared with their WT counterparts. The reasons for this phenomenon are still unclear, but we speculate that this T cell hyperactivation is due to the appearance of an abnormal CD3-CD44^{high} cell type that has features of plasma cells (G. Varga et al., unpublished observations). Currently, experiments are underway that investigate the relevance of β_2 integrins for APC-T cell interaction during antigen presentation in more detail.

Indeed, β_2 integrins may not only be critical for leukocyte-endothelial cell and leukocyte-leukocyte adhesion, but they could also contribute to regulation of T cell stimulation or homeostasis *in vivo*.

In many respects, the *CD18*-deficient mouse shares features with the *LFA-1*-mutant mouse strains generated by Schmits et al. (33). These mutant mice also display markedly reduced ACD, splenomegaly, impaired T cell proliferation after stimulation with alloantigen or anti-CD3, and defects in natural killer cell function and tumor rejection, (S. Grabbe, unpublished observations). Interestingly, Shier et al. (32) reported that another *LFA-1*-mutant mouse strain did not develop abnormalities in cell numbers or in lymphocyte subsets of lymphatic organs. We observed that the abnormalities in lymphoid organs of *CD18*^{-/-} mice developed as the mice aged, and we speculate that they may be related to an uncontrolled response to exogenous antigens (such as commensal bacteria). Thus, differences in phenotype may also be related to the timepoint of investigation and animal housing conditions.

Another surprising observation was that elicitation of irritant dermatitis evoked an unaltered tissue edema, despite markedly impaired cellular infiltration in *CD18*^{-/-} mice (Figure 4). Thus, cellular infiltration and tissue edema are separate events that are induced by different mechanisms. These data also suggest that measuring ear swelling responses to assess tissue edema, which is the most commonly used system to quantitate ACD, may not always be an effective way to quantify antigen-specific T cell-mediated immune responses *in vivo*, since tissue edema is not primarily induced by infiltrating T cells and therefore may not reflect the degree of T cell activation.

In summary, we found a complete blockade in elicitation of antigen-specific, T cell-mediated cutaneous immune responses in CD18-deficient mice, that is primarily mediated by defective T cell transmigration into inflamed tissues. Thus, these data suggest that the immunopathology of human LAD I may not only be due to a defective innate immune system (macrophages and granulocytes). A dysfunction of the acquired immune system, notably defective T cell extravasation, may contribute to many of the clinical symptoms in these patients.

Acknowledgments

The authors wish to thank M. Gunzer, A. Mehling, and D. Vestweber for helpful discussions and critical review of the manuscript. This work was supported by grants from the German Research Association to S. Grabbe (Gr1022/7-1, SFB293/B1, and SFB492/B2) and C. Sunderkötter (IZKF-C2 and SFB293/A8).

- Hynes, R.O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell*. **69**:11–25.
- Issekutz, T.B. 1992. Lymphocyte homing to sites of inflammation. *Curr. Opin. Immunol.* **4**:287–293.
- Luscinskas, F.W., and Lawler, J. 1994. Integrins as dynamic regulators of vascular function. *FASEB J.* **8**:929–938.
- Benimetskaya, L., et al. 1997. Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein. *Nat. Med.* **4**:414–420.
- El Ghmati, S.M., van Hoeyveld, E.M., van Strijp, J.A.G., Ceuppens, J.L., and Stevens, E.A.M. 1996. Identification of haptoglobin as an alternative ligand for CD11b/CD18. *J. Immunol.* **156**:2542–2552.
- Coxon, A., et al. 1996. A novel role for the $\beta 2$ integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. *Immunity*. **5**:653–666.
- Gahmberg, C.G., Tolvanen, M., and Kotovuori, P. 1997. Leukocyte adhesion—structure and function of human leukocyte beta2-integrins and their cellular ligands. *Eur. J. Biochem.* **245**:215–232.
- Kanazashi, S.I., Sharma, C.P., and Arnaout, M.A. 1997. Integrin-ligand interactions: scratching the surface. *Curr. Opin. Hematol.* **4**:67–74.
- Noti, J.D., Johnson, A.K., and Dillon, J.D. 2000. Structural and functional characterization of the leukocyte integrin gene CD11d. Essential role of Sp1 and Sp3. *J. Biol. Chem.* **275**:8959–8969.
- Anderson, D.C., and Springer, T.A. 1987. Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p159,95 glycoproteins. *Annu. Rev. Immunol.* **114**:145–181.
- Scharffetter-Kochanek, K., et al. 1998. Spontaneous skin ulceration and defective T cell function in CD18 null mice. *J. Exp. Med.* **188**:119–131.
- Grabbe, S., and Schwarz, T. 1998. Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. *Immunol. Today*. **19**:37–44.
- Grabbe, S., Steinbrink, K., Steinert, M., Luger, T.A., and Schwarz, T. 1995. Removal of the majority of epidermal Langerhans cells by topical or systemic steroid application enhances the effector phase of murine contact hypersensitivity. *J. Immunol.* **155**:4207–4217.
- Grabbe, S., et al. 1996. Dissection of antigenic and irritative effects of epicutaneously applied haptens in mice. Evidence that not the antigenic component but nonspecific proinflammatory effects of haptens determine the concentration-dependent elicitation of allergic contact dermatitis. *J. Clin. Invest.* **98**:1158–1164.
- Schwarz, A., et al. 1994. In vivo effects of interleukin-10 on contact hypersensitivity and delayed-type hypersensitivity reactions. *J. Invest. Dermatol.* **103**:211–216.
- Sunderkötter, C., et al. 1996. Activated T cells induce expression of E-selectin in vitro and in an antigen-dependent manner in vivo. *Eur. J. Immunol.* **26**:1571–1579.
- Labeur, M., et al. 1999. Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J. Immunol.* **162**:168–175.
- Inaba, K., et al. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony stimulating factor. *J. Exp. Med.* **176**:1693–1702.
- Austrup, F., et al. 1997. P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissues. *Nature*. **385**:81–83.
- Robert, C., et al. 1999. Interaction of dendritic cells with skin endothelium: new perspective on immunosurveillance. *J. Exp. Med.* **189**:627–636.
- Pendl, G., et al. 2002. Immature mouse dendritic cells enter inflamed tissue, a process that requires E- and P-selectin, but not PSGL-1. *Blood*. In press.
- Issekutz, T.B. 1995. In vivo blood monocyte migration to acute inflammatory reactions, IL-1 alpha, TNF-alpha, IFN-gamma, and C5a utilizes LFA-1, Mac-1, and VLA-4. The relative importance of each integrin. *J. Immunol.* **154**:6533–6540.
- Shang, X.Z., and Issekutz, A.C. 1998. Contribution of CD11a/CD18, CD11b/CD18, ICAM-1 (CD54) and -2 (CD102) to human monocyte migration through endothelium and connective tissue fibroblast barriers. *Eur. J. Immunol.* **28**:1970–1979.
- Shang, X.Z., Lang, B.J., and Issekutz, A.C. 1998. Adhesion molecule mechanisms mediating monocyte migration through synovial fibroblast and endothelium barriers: role for CD11/CD18, very late antigen-4 (CD49d/CD29), very late antigen-5 (CD49e/CD29), and vascular cell adhesion molecule-1 (CD106). *J. Immunol.* **160**:467–474.
- Mizger, J.P., et al. 1997. Neutrophil emigration in the skin, lungs, and peritoneum: different requirements for CD11/CD18 revealed by CD18-deficient mice. *J. Exp. Med.* **186**:1357–1364.
- Andrew, D.P., et al. 1998. Transendothelial migration and trafficking of leukocytes in LFA-1-deficient mice. *Eur. J. Immunol.* **28**:1959–1969.
- Issekutz, A.C., Chuluyan, H.E., and Lopes, N. 1995. CD11/CD18-independent transendothelial migration of human polymorphonuclear leukocytes and monocytes: involvement of distinct and unique mechanisms. *J. Leukoc. Biol.* **57**:553–561.
- Schneeberger, E.E., Vu, Q., LeBlanc, B.V., and Doershuk, C.M. 2000. The accumulation of dendritic cells in the lung is impaired in CD18^{-/-} but not in ICAM^{-/-} mutant mice. *J. Immunol.* **164**:2472–2478.
- Issekutz, T.B. 1993. Dual inhibition of VLA-4 and LFA-1 maximally inhibits cutaneous delayed-type hypersensitivity-induced inflammation. *Am. J. Pathol.* **143**:1286–1293.
- Gao, J.X., Issekutz, A.C., and Issekutz, T.B. 1994. Neutrophils migrate to delayed-type hypersensitivity reactions in joints, but not in skin. Mechanism is leukocyte function-associated antigen-1/Mac-1-independent. *J. Immunol.* **153**:5689–5697.
- Issekutz, A.C., and Issekutz, T.B. 1995. Monocyte migration to arthritis in the rat utilizes both CD11/CD18 and very late activation antigen 4 integrin mechanisms. *J. Exp. Med.* **181**:1197–1203.
- Shier, P., et al. 1996. Impaired immune responses toward alloantigens and tumor cells but normal thymic selection in mice deficient in the $\beta 2$ integrin leukocyte function-associated antigen-1. *J. Immunol.* **157**:5375–5386.
- Schmits, R., 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.
- Dustin, M.L., and Cooper, J.A. 2000. The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signaling. *Nat. Immunol.* **1**:23–29.