Leukocytosis and Resistance to Septic Shock in Intercellular Adhesion Molecule 1–deficient Mice

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Summary

Intercellular adhesion molecule 1 (ICAM-1) is one of three immunoglobulin superfamily members that bind to the integrins lymphocyte function associated 1 (LFA-1) and Mac-1 on leukocytes. We have generated mice that are genetically and functionally deficient in ICAM-1. These mice have elevated numbers of circulating neutrophils and lymphocytes, as well as diminished allogeneic T cell responses and delayed type hypersensitivity. Mutant mice are resistant to lethal effects of high doses of endotoxin (lipopolysaccharide [LPS]), and this correlates with a significant decrease in neutrophil infiltration in the liver. Production of inflammatory cytokines such as tumor necrosis factor α or interleukin 1 is normal in ICAM-1–deficient mice, and thus protection appears to be related to a diminution in critical leukocyte–endothelial interactions. After sensitization with D-galactosamine (D-Gal), ICAM-1–deficient mice are resistant to the lethal effect of low doses of exotoxin (*Staphylococcus aureus* enterotoxin B [SEB]), which has been shown to mediate its toxic effects via the activation of specific T cells. In this model, ICAM-1–mediated protection against SEB lethality correlates with a decrease in the systemic release of inflammatory cytokines, as well as with prevention of extensive hepatocyte necrosis and hemorrhage. ICAM-1–deficient mice sensitized with D-Gal, however, are not protected from lethality when challenged with low doses of endotoxin (LPS). These studies show that the different contribution of ICAM-1 in the activation of either T cells or macrophages is decisive for the fatal outcome of the shock in these two models. This work suggests that anti-ICAM-1 therapy may be beneficial in both gram-positive and -negative septic shock, either by reducing T cell activation or by diminishing neutrophil infiltration.

Adhesion receptors in the immune system control cell–cell interactions required for the activation of lymphocytes by foreign antigens, and for directing the migration and localization of leukocytes. Leukocyte interaction with blood vessel walls and transendothelial migration are necessary for efficient defense against infections and are dysregulated in harmful inflammatory processes. The interplay of adhesion molecules, namely the integrins, the Ig superfamily members, and the selectins with chemoattractants and their receptors, is thought to be critical for leukocyte extravasation. The $\beta 2$ (CD18) subfamily of integrins expressed on leukocytes is composed of three members, LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150,95 (CD11c/CD18) (1, 2). LFA-1 is expressed on all leukocytes, whereas Mac-1 and p150,95 are largely restricted to monocytes and granulocytes. LFA-1 binds to three Ig superfamily members, intercellular adhesion molecule (ICAM)[1] 1, 2, and 3 (3). All three molecules are found on leukocytes but only ICAM-1 and -2 are expressed on vascular endothelium (4–7). Mac-1 also binds to ICAM-1 (8, 9). LFA-1 binds to domain 1 of ICAM-1, whereas Mac-1 binds to domain 3 (2, 10). Previous experiments have shown that the LFA-1–dependent adhesion of lymphocytes and monocytes to resting endothelial cells in vitro is about one-third dependent on interaction with ICAM-1 and about two-thirds dependent on interaction with ICAM-2, reflecting the finding that ICAM-2 is expressed constitutively

[1] *Abbreviations used in this paper:* D-Gal, D-galactosamine; DTH, delayed type hypersensitivity; ES cell, embryonic stem cell; ICAM, intercellular adhesion molecule; LAD, leukocyte adhesion deficiency; MLR, mixed lymphocyte reaction; SEB, Staphylococcus enterotoxin B.

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on resting endothelial cells at much higher levels than ICAM-1. However, upon stimulation by inflammatory cytokines in vitro, ICAM-1 expression on endothelial cells is increased up to 40-fold whereas ICAM-2 expression is unaffected (4–6, 11). Induction of ICAM-1 results in greatly increased binding of both lymphocytes and neutrophils to endothelial cells (9, 11).

It has been hypothesized that leukocyte emigration from the circulation into surrounding tissues involves sequential events that have been described as (*a*) initial rolling of leukocytes on inflamed vascular endothelium via selectin interaction with carbohydrate ligands; (*b*) activation of leukocytes by chemoattractants; (*c*) firm attachment to the blood vessel walls mediated by interactions between integrins and their ligands; and (*d*) transendothelial migration (3, 12, 13). Disruption of this sequence at any step would presumably prevent leukocyte emigration and accumulation at inflammatory sites and would severely affect normal inflammatory responses. The critical role in inflammatory responses and immune functions of $\beta 2$ integrins, and of the carbohydrate ligands of selectins is best illustrated in two human genetic diseases, leukocyte adhesion deficiency (LAD) I and II. Whereas LAD II is caused by a block in synthesis of fucosylated carbohydrates (14), LAD I is caused by mutations in the gene encoding the $\beta 2$ (CD18) subunit used by the integrins LFA-1, Mac-1, and p150,95 (15, 16). In LAD I patients, granulocytes are unable to migrate to and accumulate at sites of infection and inflammation (17, 18), but are able to adhere and roll on blood vessel endothelium in vivo (19).

Although migration of circulating leukocytes from blood into surrounding tissues is a critical step of inflammation necessary for host defense, excessive accumulation of leukocytes can be harmful and can lead to inflammatory disorders including vasculitis, arthritis, asthma, and ischemia-reperfusion injury.

Septic shock is a systemic response to infection with high mortality (20). 70% of septic shocks in humans are caused by gram-negative bacterial endotoxin and up to 30% are caused by gram-positive bacteria. *Staphylococcus aureus* enterotoxin B (SEB) is a bacterial exotoxin from gram-positive bacteria that causes toxic shock in humans and in mice (21). The endogenous mediators TNF-α and IL-1, released in response to LPS and other products of gram-negative or-positive bacteria have been identified as the principal mediators of the pathology in sepsis. The release of these endogenous mediators leads to a number of pathophysiological reactions, such as fever, leukopenia, thrombocytopenia, disseminated intravascular coagulation, leukocyte infiltration in various organs, and hemodynamic changes that ultimately may lead to lethal shock. It has been proposed that hepatic ischemia followed by a reperfusion syndrome is what causes the irreversible liver damage in septic shock, but the mechanisms by which the release of inflammatory cytokines lead to this reperfusion injury are undetermined (22). In spite of the several adhesion-dependent phenomena that occur in septic shock, such as leukocyte activation and infiltration, no evaluation of the role of adhesion molecules in this process has been performed.

The concept of antiadhesion therapy has been validated in experimental animals by the demonstration that mAbs to integrins and selectins inhibit leukocyte-mediated damage in a wide range of inflammatory disease models (23, 24). Anti-ICAM-1 antibodies inhibit leukocyte infiltration and tissue injuries in several models of lung inflammatory disease (25, 26), as well as kidney transplant rejection (27). Potential shortcomings of evaluating the in vivo function of a molecule with inhibitory mAbs are that mAbs can have additional effects such as immune-mediated damage or elimination of cells on which the target antigen is expressed; mAbs bind to only a single epitope and may not block all adhesive interactions, especially for molecules such as ICAM-1 that have multiple integrin binding sites; and evaluation of long-term effects, such as development, is difficult.

To date, most of our knowledge about ICAM-1 has been focused on its mechanisms of action in vitro, but the contributions of ICAM-1 in various inflammatory states in vivo, the significance in vivo of redundant interactions between leukocyte integrins and their ligands, as well as the separate functions of ICAM-1, -2, and -3, remain to be fully understood. In this study, we have generated ICAM-1–deficient mice by gene targeting in embryonic stem (ES) cells (28), and we have used these mutant mice to study the specific role of ICAM-1 in septic shock.

Materials and Methods

Gene Targeting in ES Cells. The λ phage clone 26 containing a portion of the ICAM-1 gene from the AKR mouse strain (29) was a kind gift of Dr. Adrienne Brian (La Jolla Cancer Research Foundation, La Jolla, CA). A 1.2-kb HindIII fragment containing exons 4 and 5 and an 8.0-kb HindIII fragment containing exons 6 and 7 were subcloned in pBlueScript (KS). To construct the targeting vector, a 1.7-kb EcoRI/HindIII fragment carrying a polyadenylated *neomycin resistance* gene (*neo*/poly A^+) under the control of the *phosphoglycero kinase* gene (PGK) promoter (a gift from Drs. En Li and Rudolf Jaenisch, Massachusetts Institute of Technology, Cambridge, MA) was isolated from pKJ1 (30), and blunted with Klenow. This fragment was inserted into the NheI site in the fourth exon of the 1.2-kb HindIII fragment of the ICAM-1 gene. The resultant 2.9-kb HindIII fragment containing the *neo*^{r} gene was then placed upstream of the 8.0-kb HindIII fragment from the ICAM-1 gene in pBlueScript (KS). Finally, a blunted 2.7-kb EcoRI/HindIII fragment from pGEM7 (thymidine kinase [tk]) containing the HSV *tk* gene under the control of the PGK promoter was subcloned in the above construct cut with NotI and blunted with Klenow (see Fig. 1).

The J1 ES cell line, obtained from Dr. Rudolf Jaenisch (30), was routinely cultured in DMEM supplemented with 15% FCS, 0.1 mM nonessential amino acids (GIBCO BRL, Gaithersburg, MD), 0.1 mM β-ME, and antibiotics. J1 ES cells were grown on feeder layers of embryonic fibroblasts pretreated with 20 μg/ml of mitomycin C for 3–4 h. 10^3 U/ml of leukemia inhibitory factor was included in the medium during selection and cloning. ES cells (5×10^7) were transfected by electroporation (240 V and 500 μF) using 25 μg/ml of plasmid DNA linearized with PvuI, as previously described (31). Transfected cells were selected with G418 (200 μg/ml of active form) and 0.2 μM FIAU (1-[2-deoxy, 2-fluoro-β-D-arabinofuranosyl]-5-iodouracil; Bristol-Myers Squibb Pharmaceutical Research, Seattle, WA) (31). Resistant colonies were picked from day 8–10 and expanded. DNA from each resistant colony was isolated and subjected to Southern blotting to identify clones that underwent homologous recombination. The mutant allele was de-

tected by both probes I and II (see Fig. 1) on BamHI- and EcoRI-digested DNA. A total of 277 resistant clones were screened and one (clone 74) showed the expected DNA restriction pattern for homologous recombination in one allele. The DNA restriction pattern corresponding to the mutant allele was confirmed upon hybridization with a specific DNA fragment derived from the *neo*^{r} gene. The *neo*^{r} gene probe did not detect any additional band in the Southern blot, demonstrating no incidence of random integration (data not shown). Furthermore, DNA digests from clone 74 ES cells were analyzed with a probe 3′ of the *neo*^{r} gene insertion (probe III), and the restriction patterns on Southern blotting were as predicted (data not shown).

Production of Chimeric and Homozygous Mutant Mice. Clone 74 ES cells carrying one mutated allele for the ICAM-1 gene were injected into C57BL/6 blastocysts to obtain chimeric mice (32). Chimeric mice were scored by agouti coat color in a black coat color background. Germline transmission attributed to recombinant ES cells was assessed by the agouti coat color of the offspring resulting from the breeding of chimeric male mice with (C57BL/6 × DBA/2)F_1 females. DNA from tail biopsies from agouti offspring was analyzed to confirm transmission of the mutation in the ICAM-1 locus. 6 of 13 chimeric male mice transmitted the disrupted gene to their progeny. Mice heterozygous for the ICAM-1 gene disruption were intercrossed to produce homozygous mutant mice.

Hematology. Mice were bled from retro-obital plexus with heparinized capillaries. Whole blood was diluted 1:10 in 2% acetic acid and total white blood cell counts determined on a hematocytometer. Blood smears were prepared and stained with LeukoStat (Sigma Chemical Co., St. Louis, MO) to count leukocyte differentials. The absolute numbers of each leukocyte population were calculated by multiplying the total white blood cell counts by differentials.

Isolation and Stimulation of Thymocytes and Splenocytes. Thymocytes and splenocytes from wild-type and mutant mice were resuspended in RPMI medium, erythrocytes were lysed with 0.15 M NH_4Cl, 1.0 mM $KHCO_3$, and cell debris was removed by three washes. Thymocyte and splenocyte responses to mitogen were performed as previously described (33). Splenocytes or thymocytes (5×10^5) were cultured in complete RPMI medium supplemented with 10% FCS in the presence of 15 μg/ml of LPS and/or 4 μg/ml of Con A for 3 d.

Antibodies and Flow Cytometry. Cell suspensions from thymus, spleen, lymph nodes, and bone marrow of 8–12-wk-old animals were prepared free from red blood cells following standard procedures. For two- and three-color flow cytometry, cells were stained with antibodies directly conjugated with fluorochrome: anti-CD4 (H129.19), anti-CD8 (53-6.7), and anti-CD3 (YCD3-1) from GIBCO BRL; anti-B220 (RA3-6B2), anti-ICAM-1 (3E2), anti-IgM (DS-1), and anti-Gr-1 (RB6-8C5) from PharMingen (San Diego, CA). The cells were incubated at 4°C for 30 min, washed three times, and fixed in PBS/1% formaldehyde before analysis on a FACScan® instrument (Becton Dickinson & Co., Mountain View, CA). Samples for one-color analysis were stained with anti–vascular cell adhesion molecule 1 (548), anti-LFA-1 (M17/4) (34), anti-Mac-1 (M1/70) (35) and anti-ICAM-2 (IC2-3C4; Xu, H., and T. Springer, manuscript in preparation). FITC-labeled rat anti–mouse κ chain antibody (MARK) from Amac, Inc. (Westbrook, ME) was used as the secondary antibody.

Immunohistology. Animals were killed by CO_2 asphyxiation, organs were immediately removed, frozen in dry ice/2-methylpentane, and stored at −70°C. Frozen sections were prepared according to standard procedures. The frozen sections were stained with hamster anti–mouse ICAM-1 mAb 3E2, washed to remove unbound antibodies, and stained with biotinylated goat anti–hamster IgG followed by avidin-biotin-peroxidase complexes using a Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. The sections were counterstained with methyl green.

Skin Contact Sensitivity Reaction. Normal BDF_1 mice and homozygous mutant mice at 8–12 wk of age were sensitized with 100 μl of 0.2% 2,4-dinitro-1-fluorobenzene (DNFB) in 3:1 acetone/olive oil, applied evenly on a shaved area of skin on the abdomen. On day 5, sensitized mice were challenged by applying 0.2% DNFB to the right ear (10 μl on the inner side and 10 μl on the outer side of the ear) (36). The thickness of the central portion of each lobe was measured 24 h after challenge using an engineer's micrometer (The Dyer Company, Lancaster, PA).

Septic Shock. LPS from *Escherichia coli* 0127:B7, SEB, and D-galactosamine (D-Gal) were purchased from Sigma Chemical Co. In septic shock experiments, age- and body weight–matched control (BDF_1 strain, The Jackson Laboratory, Bar Harbor, ME, and wild-type littermates) and ICAM-1–deficient mice were injected intraperitoneally with 40 mg/kg LPS or with a mixture of D-Gal (20 mg/mouse) and the amounts of LPS or SEB indicated in the results section. All animals were cared for by a full-time veterinary staff and monitored daily for signs of morbidity. Two wild-type mice exhibiting painful distress (convulsions) were killed by cervical dislocation, and were excluded from the experiments. Invasive procedures were carried out under anesthesia with metafane (Pitman-Moore, Inc., Mundelein, IL).

Cytokine Measurement. The systemic release of cytokines after toxin challenge was determined by ELISA. Blood was taken at indicated time points after treatment and cytokine concentrations were measured in duplicate. Sera from two or three different animals was pooled within each group to reduce the number of samples. Two pools from each experimental and control group were measured. Serial dilutions of serum samples were assayed by ELISA for TNF-α, IL-6, and IL-1α (Endogen, Inc., Boston, MA) as recommended by the supplier. Absorbance values read at 450 nm were converted to concentrations (picograms per milliliter) in the serum by comparison with the respective standard curve.

Histology. Tissues were fixed in 10% formaldehyde, sectioned, and stained with hematoxylin and eosin. The pathohistology of the livers of D-Gal–sensitized mice challenged either with LPS or SEB was studied 10–12 h after treatment in sections of at least three different animals per group. The chloroacetylesterase histochemical procedure was used to aid in the visualization of neutrophils. For the evaluation of neutrophil infiltration in the high-dose LPS model, neutrophils in liver sections in five mutant (−/−) and four wild-type (+/+) mice were quantitated by counting the total number of neutrophils, as well as the number of small (two to five) and large (over five) neutrophil clusters in eight high-power fields (at a magnification of 40; total area 0.5 mm^2), which were selected randomly at a low-power magnification of 4 at which neutrophils are not visible.

Results

Disruption of the ICAM-1 Gene and Generation of ICAM-1–deficient Mice. The mouse ICAM-1 gene consists of seven exons (29, 37). Exon 1 encodes the 5′ untranslated region and signal peptide, exons 2–6 encode the five Ig-like domains, and exon 7 encodes the transmembrane and the cytoplasmic domains. To disrupt the ICAM-1 gene by homologous recombination, a replacement vector was made (28) with a *neo*^{r}

cassette inserted in exon 4 (Fig. 1 *A*). The targeting construct also contained the *tk* gene to allow negative selection in screening for homologous recombination (28, 31).

ES cells of the J1 line were transfected with the targeting vector and selected for resistance to G418 and FIAU (30). Southern blots of DNA from resistant colonies showed homologous recombination in ES cell clone 74. The fidelity of homologous recombination in clone 74 was confirmed with probes on both sides of the replacement vector integration site. ES cells from clone 74 were expanded and microinjected into C57BL/6 blastocysts to make chimeric mice. Six independent fertile chimeras transmitted the mutant allele to the offspring. Two of these lines were propagated and used for further analysis. A representative Southern blot of a litter resulting from intercross between mice heterozygous for the ICAM-1 mutation is shown in Fig. 1 *B*. Three of the offspring were heterozygotes and showed both a 3.8-kb BamHI DNA fragment from the wild-type allele and a 2.7-kb BamHI band corresponding to the mutant allele. Two of the offspring showed only the band from the mutant allele and, therefore, were homozygous for ICAM-1 disruption. The predicted frequency of homozygous mutant animals was obtained both inside and outside a specific pathogen-free animal facility. Mice homozygous for ICAM-1 gene disruption did not show any gross abnormality in development or fertility.

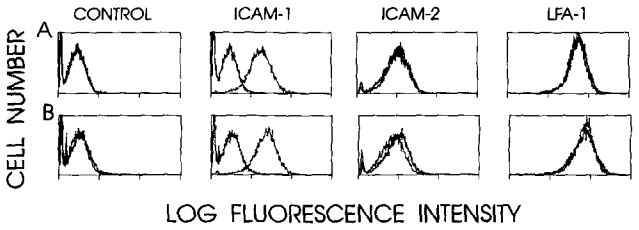

Figure 2. Expression of adhesion molecules on mitogen-stimulated lymphoblasts. Con A–stimulated thymocytes (*A*) or Con A and LPS-stimulated spleen cells (*B*) from wild-type (*thin line*) or ICAM-1–deficient (*thick line*) mice were stained with mAbs to the indicated adhesion molecules or control ascites and subjected to flow cytometry.

The Lack of ICAM-1 in Homozygous Mutant Mice Does Not Affect Lymphoid Development but Results in Reduced Antigen-specific Immune Responses. ICAM-1 expression is very low on resting lymphocytes but is greatly increased upon stimulation by mitogens (38). Therefore, thymocytes and splenocytes isolated from normal and homozygous mutant mice were treated for 72 h with ConA and with a combination of ConA and LPS, respectively, before being examined for expression of ICAM-1 by immunofluorescence and flow cytometry (Fig. 2). Mitogen-stimulated thymocytes and splenocytes from wild-type control mice showed high ex-

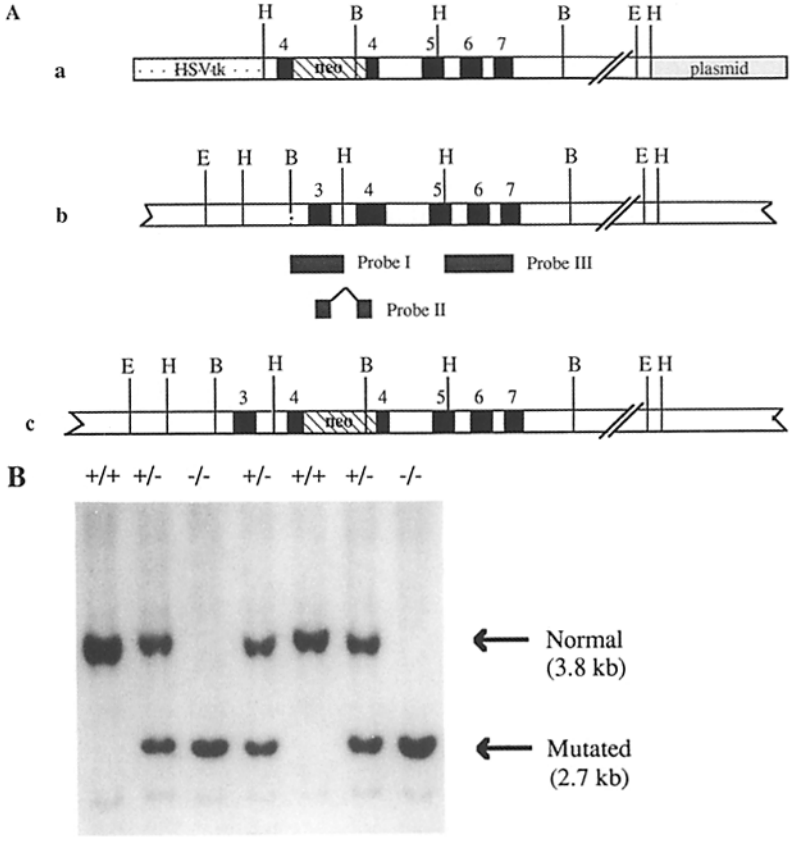

Figure 1. Design of the replacement vector and Southern blots showing mutant allele segregation in a representative litter. (*A*) Strategy for homologous recombination. (*a*) The replacement vector contains a PGK-*neo*^{r} gene cassette inserted in the exon 4. The *neo*^{r} gene insertion was flanked by 0.7- and 8.0-kb DNA from the ICAM-1 locus. (*b*) Restriction map of the wild-type ICAM-1 locus. (*c*) Restriction map of the mutated ICAM-1 allele after homologous recombination. The locations of probe I (a BamHI/HindIII genomic DNA fragment), probe II (an NheI cDNA fragment) and probe III (an NcoI/SalI genomic fragment) are indicated. Exons are shown in black and are numbered. Restriction sites listed are: (*B*) BamHI; (*E*) EcoRI; and (*H*) HindIII. (*B*) Southern blots of tail DNA isolated from a litter of seven mice from a heterozygous intercross. Tail DNA was digested with restriction enzyme BamHI, electrophoresed, blotted, and hybridized with probe I. The fragments detected by the probe from the normal and mutated allele are indicated. (*Top*) The genotypes of individual mice.

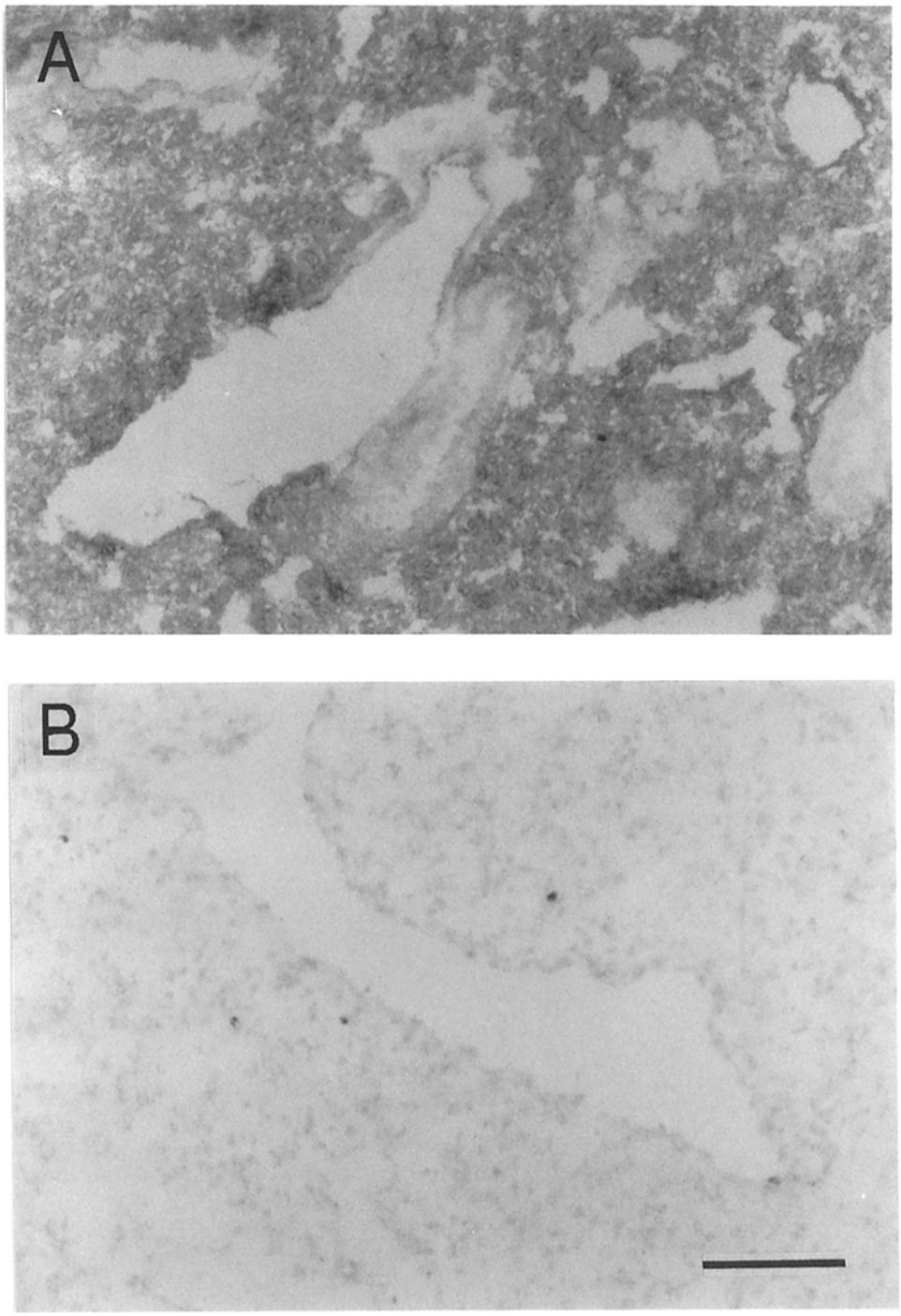

Figure 3. ICAM-1 expression in lung. Frozen sections of lungs from wild-type (*A*) and ICAM-1–deficient (*B*) mice were stained with anti-ICAM-1 mAb and peroxidase and then counterstained with methyl green. ICAM-1 expression appears as black staining in the light gray background. Black spots (*B*) are due to background peroxidase activity. Bar, 200 μm.

pression of ICAM-1 whereas cells from the homozygous mutant mice showed no expression of ICAM-1. Wild-type and mutant cells expressed equivalent levels of LFA-1 and ICAM-2. ICAM-1 was also absent on primary keratinocytes isolated from mutant mice as shown by flow cytometry, whereas normal keratinocytes expressed low levels of ICAM-1 (data not shown).

ICAM-1 has been shown previously to be expressed on a wide variety of nonhematopoeitic cells (4, 6). Immunohistochemical staining of frozen sections of wild-type mice showed diffuse ICAM-1 positive staining in lung on alveolar epithelial cells, in blood vessel endothelium, and in certain interstitial areas (Fig. 3). In the thymus, ICAM-1 was expressed on cortical and medullary thymocytes and on thymic epithelial cells (data not shown). By contrast, mutant mice were completely negative for ICAM-1.

The distribution of ICAM-1 on thymic epithelial cells in fetal and adult thymus and on germinal center dendritic cells

has led to the hypothesis that ICAM-1 might play a role in thymocyte maturation and in the formation of germinal centers. However, no detectable differences in the absolute numbers of major T and B cell subsets were observed in the thymus, spleen, and lymph nodes between wild-type and ICAM-1–deficient mice (data not shown). The only detectable cellular difference between ICAM-1–deficient and wild-type mice was a significant elevation in circulating neutrophils and lymphocytes (see below).

To investigate whether there was any defect in lymphocyte function, we performed mixed lymphocyte reaction (MLR) assays to measure T cell proliferation in response to allogeneic cells. These experiments showed that lymphocytes from ICAM-1–deficient mice are not hindered as responders, but are diminished in their ability to stimulate an allogeneic response (data not shown).

Administration of antibodies against LFA-1 and ICAM-1 in mice partially inhibits the delayed-type hypersensitivity (DTH) reaction induced by DNFB (39). Anti-LFA-1 inhibits the reaction more substantially than anti-ICAM-1. In this model of in vivo T cell responses to specific antigen, ICAM-1–deficient mice showed a significant reduction in ear swelling as the hapten-specific reaction was reduced by ~50% (Table 1).

ICAM-1–deficient Mice Are Resistant to the Lethal Effect of High Doses of Endotoxin but Do Not Show Decreased Production of TNF-α and IL-1. Lethal endotoxin shock was induced by intraperitoneal injection of high doses of LPS (40 mg/kg) in mice at 8–14 wk of age. Normal mice demonstrated a series of responses including shivering, a sign of fever, lethargy, watery eyes due to enhanced vasopermeability, and ultimately, death. Only 21% of the wild-type mice survived (4 of 19) and the deaths occurred 24–48 h after receiving LPS (Fig. 4). Similar symptoms of endotoxin shock were observed in ICAM-1–deficient mice, but the animals were less lethargic. Most importantly, the vast majority (22 of 23 animals in experiment 1) of ICAM-1–deficient mice survived LPS shock (Fig. 4). It thus appears that cell–cell interaction via ICAM-1 is an important mechanism in the sequence of events that lead to lethality during endotoxin shock.

Administration of LPS to mice induces production and secretion, largely by macrophages, of the proinflammatory cytokines TNF-α, IL-1, and IL-6, which are released sequentially into the circulation (40). The peak concentrations for these cytokines in the serum are detected between 1 and 4 h after LPS administration, reaching a plateau level after this period. To examine whether ICAM-1 mutant mice have altered serum levels of inflammatory cytokines, we have followed the kinetics of systemic TNF-α, IL-1, and IL-6 release in ICAM-1 mutant and wild-type mice after challenge with a lethal dose of LPS. No significant differences in the circulating levels of TNF-α or IL-1 were observed between mutant and wild-type mice (Fig. 5 *A*). Similar results were obtained for IL-6 serum levels (data not shown).

Leukocyte counts were monitored during LPS shock (Fig. 5 *B*). Baseline white blood cell counts were severalfold higher in ICAM-1–deficient mice. Neutrophils were two to six and lymphocytes were two to three times higher in ICAM-1–deficient mice, even though there was no sign of infection and mice were specific pathogen-free. 2 h after LPS injection, there was a drastic decline of circulating neutrophil and lymphocyte counts in both wild-type and ICAM-1–deficient mice. The circulating neutrophil counts in ICAM-1–deficient mice dropped to a similar level to those of wild-type mice at 2 h, but rose back to an elevated level at 6 h after LPS treatment (Fig. 5 *B*). By contrast, the LPS-induced lymphopenia was sustained in both ICAM-1–deficient and wild-type mice (Fig. 5 *B*).

Leukocyte accumulation and infiltration were examined histologically in organs from mice killed at 2 and 24 h after LPS treatment. The most significant histopathologic difference between wild-type and deficient mice was in the liver 24 h after LPS treatment (Table 2 and Fig. 6). The liver of each LPS-treated wild-type mouse showed diffuse infiltration with leukocytes, predominantly neutrophils. Neutrophils were present as single or rows of cells within hepatic sinusoids, or as clusters of neutrophils within the hepatic cords, often

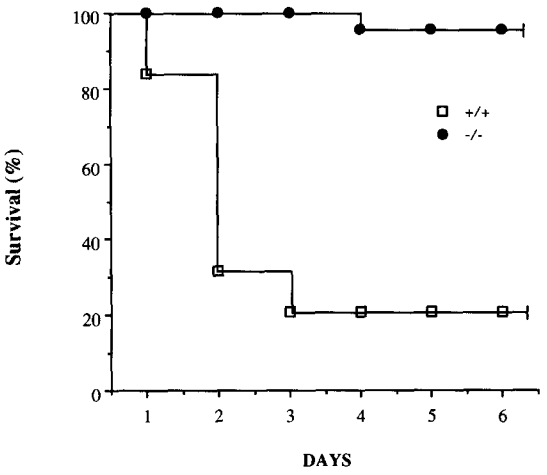

Figure 4. Survival of mice treated with a lethal dose of LPS. Mice were given 40 mg/kg of LPS i.p. on day 0. Survival data are shown from three independent experiments, with age- and body weight–matched mice, with 19 wild-type (+/+, *open square*) and 23 mutant (−/−, *filled circle*), housed both inside and outside a specific pathogen-free facility. p value for the difference in the survival rate was <0.005, tested by χ^2.

Table 1. *Reduced Contact Sensitivity Reaction in ICAM-1–deficient Mice*

Genotype	Ear thickness (Mean μm ± SD)		
	Unchallenged ear	Challenged ear	Increase in ear thickness*
+/+ (n = 5)	220 ± 8.9	442 ± 36.6	222 ± 31.9
−/− (n = 6)	228 ± 13.4	338 ± 42.2	110 ± 50.3

* p <0.005 (by Student's t test).

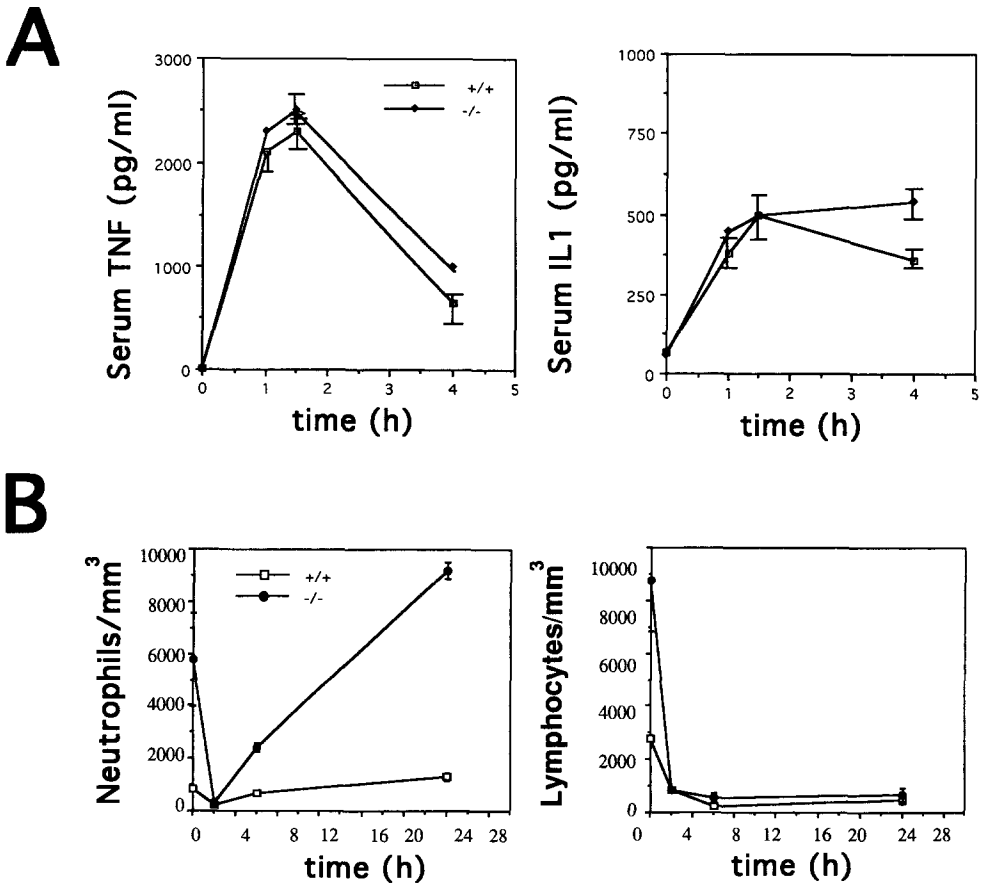

Figure 5. Systemic release of cytokines (*A*) and peripheral blood neutrophil and lymphocyte counts (*B*) before and after administration of high doses of LPS. (*A*) Wild-type or ICAM-1 mutant mice (four to six per group) were bled from the retro-obital plexus with heparinized capillaries at indicated times after injection of LPS. At different time points, different groups of animals were used. Within each group, two pools of sera from two to three animals each were measured by ELISA in duplicate. One representative experiment out of two is shown. (*B*) Mice (four to six per group) were bled from the retro-obital plexus with heparinized capillaries before or at 2, 6, and 24 h after intraperitoneal injection of LPS. Total and differential white blood cell counts from individual mice were determined, and the absolute numbers of neutrophils (*A*) and lymphocytes (*B*) were calculated (shown as the mean ± SD).

Table 2. *Quantitation of Neutrophils in Liver Sections Obtained 24 h after Induction of High-dose LPS Septic Shock*

		Number of neutrophils (per 0.5 mm²)*		
		Clusters‡		
	Genotype	2–5	>5	Total§
	+/+	23	14	165
	+/+	27	8	163
	+/+	29	14	187
	+/+	23	14	169
Mean ± SD		25.5 ± 2.6	12.5 ± 2.6	171 ± 9.5
	−/−	2	0	64
	−/−	1	0	54
	−/−	4	1	63
	−/−	4	4	104
	−/−	2	1	61
Mean ± SD		2.6 ± 1.3‖	1.2 ± 1.6¶	69.2 ± 19.8**

* Fields were selected at low-power (4×) magnification to ensure random selection and quantitated at 40X magnification.
‡ Number of clusters per 0.5 mm² containing two to five or more than five cells per cluster.
§ Total neutrophils within clusters plus those present as single cells.
‖ $p = 0.0008$ (by Student's t test).
¶ $p = 0.0025$ (by Student's t test).
** $p = 0.0001$ (by Student's t test).

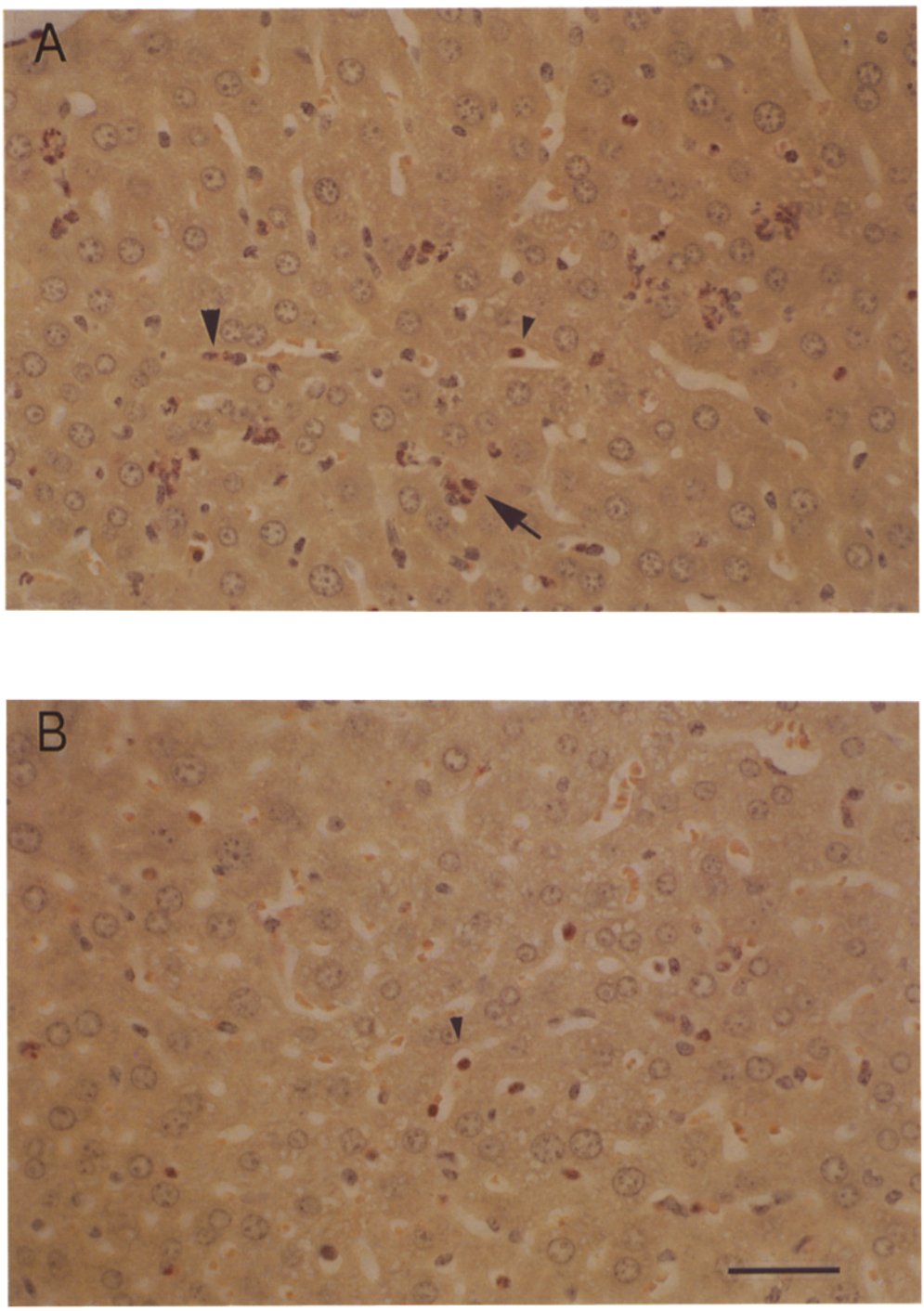

Figure 6. Representative liver sections from mice during high-dose endotoxin shock. Livers from wild-type (*A*) and ICAM-1–deficient (*B*) mice that were killed 24 h after intraperitoneal injection of LPS were fixed in 10% formalin, sectioned, and stained with chloroacetylesterase to visualize neutrophils. The arrows and arrowheads indicate neutrophils, which are present as single cells (*small arrowheads*), as rows of cells within the sinusoids (*large arrowhead*), or as small or large cellular clusters within the hepatic parenchyma (*arrow*). Bar, 100 μm.

obscuring hepatic cells. The majority of clusters were made up of two to five neutrophil profiles, but larger accumulations of up to 10 nuclei and occasional aggregates of more than 10 nuclei were present. Although the severity of the infiltrate varied in areas of the liver among individual animals, neutrophil clusters were present in virtually all fields of all four wild-type livers examined at 24 h after LPS injection. In contrast, the livers of ICAM-1–deficient mice showed significantly fewer clusters and aggregates (Table 2), and areas of the liver with clusters were scattered and small. Intrasinusoidal neutrophils, however, were present and in many areas appeared greater in number over wild-type liver, reflecting the marked increase in peripheral neutrophil counts in the ICAM-1 mutant mice (Fig. 5 *B*). We could not unequivocally document differences in neutrophil infiltration in the lung and spleen sections. There was little neutrophil influx in the kidney and heart in either group. It is interesting to note that the livers of mice of both groups treated with LPS

Table 3. *Mortality of ICAM-1–deficient Mice in Several Septic Shock Models*

Genotype	D-Gal (mg, i.p.)	LPS (μg, i.p.)	SEB (μg, i.v.)	Lethality (death/total)
+/+	20	10	–	3/3
+/+	20	10	–	4/5
+/+	20	50	–	3/3
–/–	20	10	–	4/4
–/–	20	10	–	2/4
–/–	20	50	–	6/6
+/+	20	–	50	4/4
+/+	20	–	50	3/3
+/+	20	–	50	4/5
–/–	20	–	50	1/6
–/–	20	–	50	1/5
–/–	20	–	50	0/4
+/+	20	–	–	0/4
–/–	20	–	–	0/6
+/+	–	–	50	0/4
–/–	–	–	50	0/6
+/+	–	10	–	0/7
–/–	–	10	–	0/6
+/+	–	40 mg/kg	–	9/10
–/–	–	40 mg/kg	–	1/12

for 2 h had many neutrophils in the sinusoids with no significant differences in numbers.

D-Gal–sensitized ICAM-1–deficient Mice Are Resistant to the Lethal Effect of Low Doses of Exotoxin (SEB) but Not to Low Doses of Endotoxin (LPS). Although mice are more resistant than humans to the pathogenic effects of bacterial toxins, septic shock can be induced by low doses of endotoxin or exotoxin after sensitization with D-Gal. D-Gal sensitizes mice to the lethal effect of toxins by lowering the threshold of hepatocyte susceptibility to the toxic products of macrophages and/or T cells (41). Mice sensitized with D-Gal and challenged at the same time with a low dose of LPS (10 or 50 μg) or SEB (50 μg) die with liver failure (41, 42).

Groups of mutant or wild-type mice received a dose of 20 mg i.p. of D-Gal, followed immediately by the injection of a low dose of LPS or SEB (Table 3). Animals receiving PBS, D-Gal, SEB, or LPS alone served as controls. In our study, D-Gal sensitization and challenge with SEB sufficed to cause death in virtually all wild-type mice in three independent experiments (Table 3). It is striking that SEB challenge of D-Gal–sensitized ICAM-1 mutant mice did not result in death (87% survival; Table 3). In contrast, sensitized ICAM-1–deficient mice showed the same mortality rate as the wild-type group when challenged with LPS. Thus, ICAM-1 deficiency protected D-Gal–sensitized mice challenged with SEB but not with LPS. In accordance with published data (41, 42), control wild-type or mutant unsensitized animals injected with SEB (up to 100 μg) or LPS (up to 50 μg) showed only marginal signs of disease such as immobility and rough fur, but not lethality.

Histopathological analysis was performed on three livers taken from animals 10 h after administration of SEB or LPS and D-Gal (Fig. 7). Sections taken from the liver tissue of wild-type or ICAM-1 mutant mice coinjected with D-Gal and LPS showed massive, widespread hepatic cell necrosis and extensive parenchymal hemorrhage. The hepatic cells that were not necrotic showed cytoplasm vacuolization, "nuclear fragmentation," karyolysis, and membrane ruptures (Fig. 7, *C* and *D*). However, the liver sections of D-Gal–sensitized mice injected with SEB showed significant differences between wild-type and mutant mice. Thus, in the wild-type animals there were numerous apoptotic cells singly (single cell necrosis) or in small clusters throughout the hepatic parenchyma, as well as areas of patchy necrosis and neutrophil infiltration with minimal or no hemorrhage (Fig. 7 *A*). In contrast, there were appreciably fewer apoptotic cells and no areas of necrosis

in ICAM-1 mutant mice coinjected with D-Gal and SEB (Fig. 7 *B*). The liver of one out of four mice from the latter group showed neutrophil clusters such as those described above.

The basic difference between these two D-Gal septic models is that macrophages are the main mediators of the reaction triggered by endotoxin (43), while the lethal shock triggered by SEB is mediated by T cells (42). Nevertheless, TNF-α, presumably produced by these target cells, is viewed as the principal mediator in both types of shock (20, 42, 44). We studied the kinetics of systemic TNF-α and IL-1 release up to 5 h after coinjection of SEB or LPS with D-Gal in wild-type and ICAM-1–deficient mice. As shown in Fig. 8, marked differences in TNF and IL-1 levels were observed between D-Gal–sensitized ICAM-1–deficient and wild-type mice challenged with SEB. Thus, TNF-α and IL-1 levels 90 min after treatment were reduced by >50% in ICAM-1–deficient mice when compared with wild-type controls. More importantly, lower systemic levels of these two cytokines were sustained in the ICAM-1 mutant for up to 8 h after treatment (data not shown). This difference was specially marked for IL-1, which after 3 h was virtually undetectable in the serum of exotoxin-treated ICAM-1–deficient mice. In contrast, only small differences were detected in the systemic levels of TNF-α or IL-1 during the first 3 h, and no differences were observed 3 h after LPS and D-Gal treatment.

Discussion

Our study shows that ICAM-1 is not essential for development and leukocyte maturation, plays an important role in antigen-specific immune functions, and is critical for mortality in septic shock. The mutation that we have introduced inactivates the ICAM-1 gene, creating mice that completely lack ICAM-1 on leukocytes and on endothelial and epithelial cells in various tissues. The phenotype of these ICAM-1–deficient mice can be exclusively attributed to the absence of ICAM-1. An alternative ligand for LFA-1 that is constitutively expressed on endothelial cells and lymphocytes, ICAM-2, remains present.

Defective Immune Responses and Leukocytosis in ICAM-1–deficient Mice. The increase in circulating neutrophils in

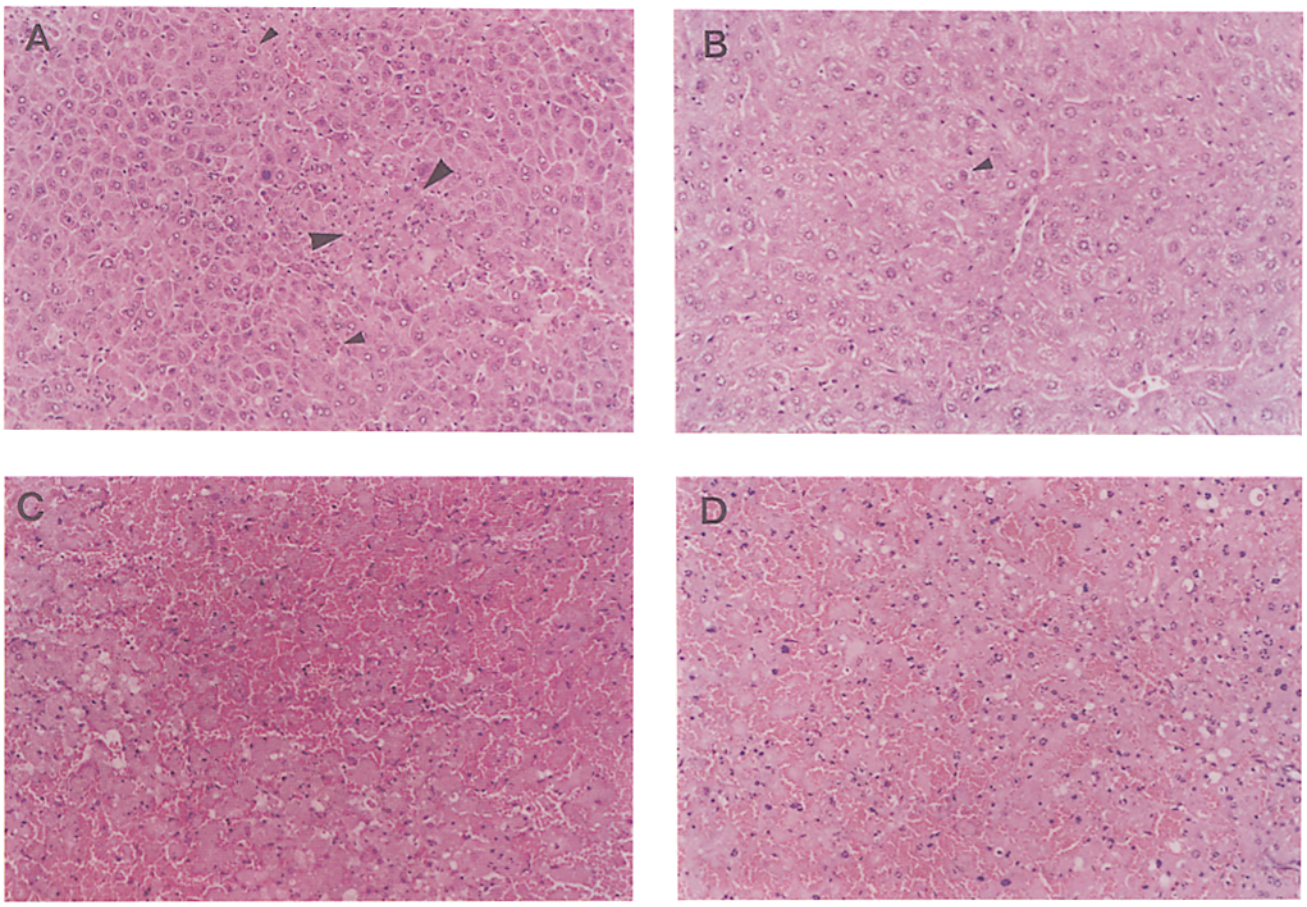

Figure 7. Representative liver sections from D-Gal–sensitized mice challenged with LPS or SEB. Livers from wild-type (*A* and *C*) and ICAM-1–deficient (*B* and *D*) mice that were killed 12 h after coinjection of D-Gal with SEB (*A* and *B*) or D-Gal with LPS (*C* and *D*) were fixed in 10% formalin, sectioned, and stained with hematoxylin-eosin. Note that after D-Gal/SEB treatment, areas of hepatic cell necrosis (*large arrowheads*) appear in wild-type livers (*A*), and are absent in ICAM-1–deficient livers (*B*). In addition, fewer apoptotic (single cell necrosis) cells (*small arrowheads*) are observed in the mutant. In contrast, after D-Gal/LPS treatment, extensive necrosis and hemorrhage are present in both wild-type (*C*) and mutant mice (*D*).

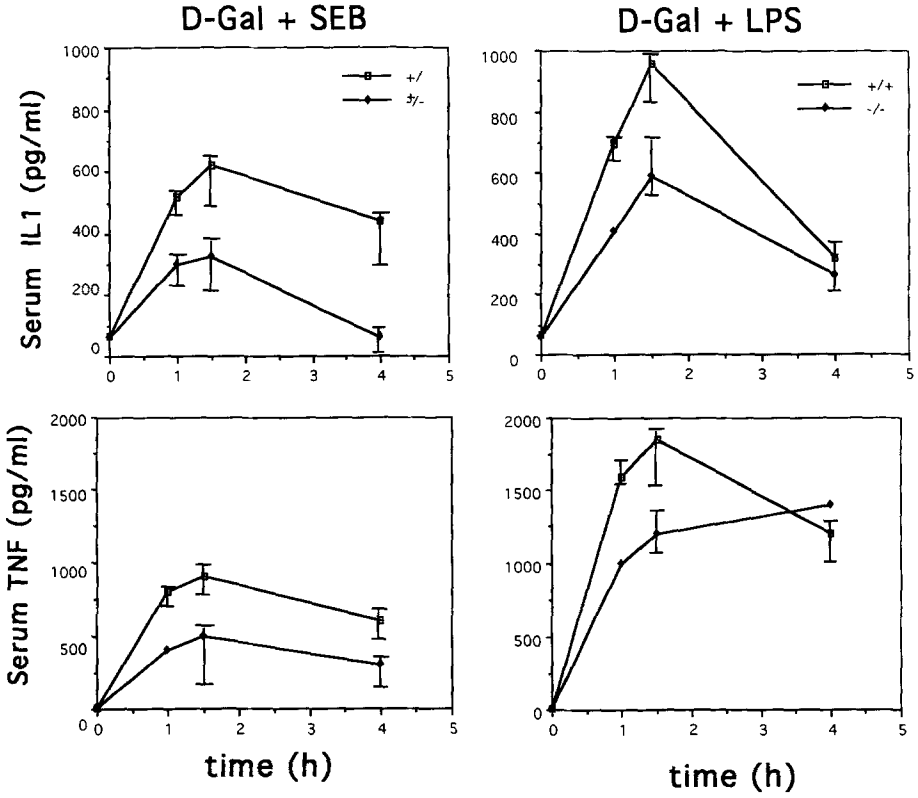

Figure 8. Kinetics of systemic cytokine release in D-Gal-sensitized mice after challenge of LPS or SEB. Wild-type and ICAM-1-deficient mice (four to six per group) were bled from the retro-obital plexus with heparinized capillaries at indicated times after coinjection of D-Gal and LPS or SEB. The bleeding was performed under anesthesia and after the procedure the mice were killed. At different time points, different groups of animals were used. Within each group, two pools of sera from two to three animals each was measured by ELISA in duplicate. One representative experiment out of three is shown.

ICAM-1-deficient mice resembles leukocytosis in LAD I patients that are deficient in $\beta 2$ integrins. However, ICAM-1 deficiency results in a modest elevation of circulating neutrophil counts (two to six times normal) compared with the 5–20-fold elevation of neutrophil counts in LAD I patients (15, 16). This may be related to the partial as compared to the complete absence of interactions dependent on the $\beta 2$ leukocyte integrins, in ICAM-1 deficiency and LAD I, respectively. Similarly, there is a relatively low elevation (2.4-fold) of circulating neutrophils in P-selectin-deficient mice (45) compared with LAD II, in which a primary defect in fucose synthesis results in a lack of carbohydrate ligands for both P- and E-selectin (14, 46). The degree of peripheral blood granulocytosis may reflect the severity of the impairment of leukocyte adherence and extravasation.

Previous studies using thymic organ culture showed that the presence of a blocking mAb to ICAM-1 affected T cell maturation in vitro (47). In this system, the effect of blocking ICAM-1 during T cell differentiation resulted in the accumulation of $CD4^-CD8^-$ thymocytes and a delay in the generation of $CD4^+CD8^+$ cells. However, our characterization of ICAM-1-deficient mice showed normal distribution of major thymocyte subsets and demonstrates that ICAM-1 is not absolutely required for thymocyte development in vivo.

This study reveals that ICAM-1 is necessary to achieve optimal antigen-specific immune functions. MLR experiments show the requirement for ICAM-1 on the APCs to reach full T cell activation, reinforcing the importance of adhesion between T cells and APCs. These findings are in agreement with previous studies that have demonstrated by mAb blocking and gene transfection that ICAM-1 expression on APCs lowers the concentration of specific antigen required to stimulate T cell responses (48–52), and that LFA-1 on T cells contributes to antigen-specific responses (48, 53–55).

We have also shown that hapten-induced contact sensitivity was reduced in ICAM-1-deficient mice. Previously, antibodies to CD11b, LFA-1, and ICAM-1 have been shown to inhibit different murine models of DTH (40, 56). Our study, which shows a 50% reduction of contact sensitivity in ICAM-1-deficient mice, parallels the amount of inhibition seen with anti-ICAM-1 mAb. The residual response may be due to the presence of another adhesion ligand for LFA-1, namely ICAM-2. An independent study on ICAM-1-deficient mice (57) showed reduced antigen-specific immune responses including MLR and DTH, and leukocytosis. These results are in good agreement with those in our study.

Role of ICAM-1 in Septic Shock. The major finding of this study is that ICAM-1 deficiency renders mice resistant to high-dose endotoxin lethal shock. The mechanism underlying this protection appears to be distal to the event triggering cytokine production, since TNF-α and IL-1 levels were similar in wild-type and ICAM-1-deficient mice. Macrophages appear to be essential in mediating endotoxin reactions, since adoptive transfer of endotoxin-sensitive macrophages renders previous endotoxin-resistant mice sensitive to endotoxin (43). Further, it has been established that the macrophage product TNF-α is central in causing endotoxin lethality (44). This cytokine, in addition to IL-1, plays a central role in the increased leukocyte adhesion, aggregation, activation, as well as in the diffuse intravascular coagulation that characterize

the shock syndrome (58–62). Intravenous administration of LPS or TNF-α produce an early and pronounced decline in peripheral blood neutrophil counts, which is largely accounted for by margination and aggregation of neutrophils in the microvasculature. During the process of neutrophil activation and adherence to endothelium, and accumulation in tissue, neutrophils may release free oxygen metabolites and various proteases that damage endothelial cells and tissues (59, 61, 62). Furthermore, leukocyte adhesion and aggregation, as well as intravascular coagulation and thrombosis, lead to occlusion of microvasculature and local ischemia. Neutrophil depletion protects mice from endotoxin shock (61). Our findings are all the more impressive because ICAM-1–deficient mice have elevated numbers of circulating neutrophils.

ICAM-1–deficient mice clearly showed responses to high doses of LPS, including shivering, lethargy, and watery eyes. Blood neutrophil counts of ICAM-1–deficient mice declined as rapidly as those of normal mice within the first 2 h after administration of LPS. Furthermore, normal and ICAM-1–deficient livers had approximately similar numbers of neutrophils at 2 h, as judged histologically. However, at 24 h, there were distinctly fewer neutrophils infiltrating the liver in all ICAM-1–deficient mice, compared with wild-type controls. These observations suggest that ICAM-1 deficiency does not affect the initial sequestration of neutrophils that causes the immediate decline in circulating neutrophils. This sequestration, which is especially prominent in the lung, results from increased viscosity of activated neutrophils, which prevents the deformation required for passage through capillaries (63, 64). The significant decrease in neutrophil infiltration in the liver at 24 h can be interpreted as reflecting deficient adhesion strengthening and transmigration, normally mediated by ICAM-1 binding to Mac-1 and LFA-1. This is supported by studies of Sligh et al. (57) demonstrating that, in ICAM-1–deficient mice, neutrophil accumulation in the peritoneal cavity in response to sterile inflammatory stimuli was diminished. We propose that the ICAM-1 protection in this model operates via abrogation or diminution of leukocyte–endothelial interactions, lowering emigration of leukocytes into tissues as well as the release of leukocyte proteases and toxic metabolites that cause tissue damage or vasoactive mediators that cause ischemia. This mechanism of protection is supported by our own results showing high TNF-α serum levels in protected ICAM-1–deficient mice, as well as by the results reported by Rothe et al. (65), who showed that TNF receptor–deficient mice are resistant to a low-dose LPS toxic shock (see below), but are highly susceptible to a high-dose LPS toxic shock. Another potential mechanism of protection would be decreased intravascular coagulation, perhaps associated with a role of ICAM-1 as fibrinogen receptor (66).

Septic shock can be induced by both gram-negative and -positive bacteria, although the latter are responsible for only 25% of the reported cases. Bacterial endotoxin is recognized as the major factor in the pathogenesis of gram-negative septic shock (20), whereas exotoxins from gram-positive bacteria cause toxic shock in humans and in mice (67). Exotoxins exhibit properties of superantigens thereby stimulating in vivo and in vitro massive T cell proliferation. The basic difference in the mechanism of action in vivo between endotoxins and exotoxin is that the endotoxin reactions are mediated by macrophages whereas the lethal shock triggered by the superantigen (i.e., exotoxin SEB) is mediated by SEB-reactive T cells.

Because mice are relatively resistant to bacterial toxins, the effects of a high dose of toxin in a murine model might resemble a substantially lower dose in human shock (42). To study the role of ICAM-1 in low-dose toxic shock models, we took advantage of the heightened sensitivity to bacterial toxins of mice treated with D-Gal to impair liver metabolism (41).

In contrast to the treatment with high doses of LPS in unsensitized mice, D-Gal–sensitized ICAM-1–deficient mice are not resistant to low doses of LPS. After D-Gal treatment, hepatocytes are severalfold more sensitive to endogenous mediators (TNF and IL-1) released after macrophage activation by LPS or by SEB-activated T cells (41). As a consequence, reperfusion injury and extensive hepatocyte necrosis occurs, followed by death (22, 68). The importance of cytokine- rather than leukocyte-dependent damage in this model is confirmed by our finding that cytokine production and lethality in ICAM-1–deficient mice are unaffected. This view is further supported by the complementary phenotype observed in TNF receptor–deficient mice (65). However, in the D-Gal/SEB–treated group, the ICAM-1 deficiency results in significantly lower levels of TNF, as well as in maintained decreased levels of IL-1, which is virtually absent 3 h after treatment. Since in mice the lethal effect of TNF-α is potentiated by IL-1 (40), and fatal outcome in human septic shock is associated with high levels of not only TNF-α, but also IL-1 (67, 69), the markedly decreased levels of IL-1 in SEB/D-Gal–treated ICAM-1–deficient mice might be responsible, together with the decrease in TNF levels, for the resistance to lethality. The reduced levels of TNF and IL-1 probably reflect directly, or as a secondary event, diminished T cell responses to SEB in vivo in ICAM-1–deficient mice. In fact, the response of Vβ8 T cells (SEB reactive) to SEB is diminished from 40 to 60% in ICAM-1–deficient mice both in vivo and in vitro (Gonzalo, J. A., unpublished results). In addition, other T cell cytokines such as IFN-γ, mediators in the hypersensitivity to toxins, are likely to be reduced in ICAM-1–deficient mice.

Thus, macrophage activation by LPS is not affected by the ICAM-1 deficiency, whereas T cell activation by SEB is critically diminished and leads to protection in D-Gal–sensitized mice. These results are in agreement with those obtained from experiments in which ICAM-1–deficient mice were infected with *Listeria monocytogenes*. These experiments revealed that the T cell-independent phase of the infection was unaffected in ICAM-1–deficient mice, whereas the T cell–dependent phase may occur at a slower rate in ICAM-1 mutant mice (Rogers, H., and E. Unanue, personal communication).

This study shows that ICAM-1 is critically involved in the massive T cell activation that occurs in sepsis by gram-positive exotoxins. ICAM-1 also is involved in leukocyte–endothelial interactions, which are central in the lethal shock induced by high doses of gram-negative endotoxins.

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