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Gab3-Deficient Mice Exhibit Normal Development and Hematopoiesis and Are Immunocompetent

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Gab proteins are intracellular scaffolding and docking molecules involved in signaling pathways mediated by various growth factor, cytokine, or antigen receptors. Gab3 has been shown to act downstream of the macrophage colony-stimulating factor receptor, c-Fms, and to be important for macrophage differentiation. To analyze the physiological role of Gab3, we used homologous recombination to generate mice deficient in *Gab3*. Gab3$^{-/-}$ mice develop normally, are visually indistinguishable from their wild-type littermates, and are healthy and fertile. To obtain a detailed expression pattern of Gab3, we generated Gab3-specific monoclonal antibodies. Immunoblotting revealed a predominant expression of Gab3 in lymphocytes and bone marrow-derived macrophages. However, detailed analysis demonstrated that hematopoiesis in mice lacking *Gab3* is not impaired and that macrophages develop in normal numbers and exhibit normal function. The lack of *Gab3* expression during macrophage differentiation is not compensated for by increased levels of Gab1 or Gab2 mRNA. Furthermore, *Gab3*-deficient mice have no major immune deficiency in T- and B-lymphocyte responses to protein antigens or during viral infection. In addition, allergic responses in *Gab3*-deficient mice appeared to be normal. Together, these data demonstrate that loss of *Gab3* does not result in detectable defects in normal mouse development, hematopoiesis, or immune system function.

Gab3 (Grb2-associated binder 3) is the most recently described member of a family of scaffolding and docking molecules, which includes two further mammalian proteins, Gab1 and Gab2, the *Drosophila melanogaster* homolog DOS (Daughter of Sevenless), and the *Caenorhabditis elegans* homolog Soc1 (suppressor of clear 1) (27). Common features of the Gab molecules are a highly conserved N-terminal pleckstrin homology domain, a central proline-rich domain, and multiple tyrosines within potential binding motifs favored by various Src homology 2 (SH2) domain-containing proteins. Upon stimulation of a variety of growth factor, cytokine, or antigen receptors, these tyrosines become phosphorylated, resulting in a transient interaction of Gab proteins with other intracellular signaling molecules. In several studies, signaling by Gab family proteins has been linked to differentiation processes. Studies on *Drosophila* mutants revealed that DOS-mediated signaling is essential for normal development of the fly (3, 7, 13, 34). Epistasis analyses have shown that the Sevenless receptor tyrosine kinase (RTK) in the fly compound eye is upstream of DOS and the protein tyrosine phosphatase Corkscrew (the *Drosophila* homolog of mammalian SHP2) can interact with and dephosphorylate activated DOS (13, 34). It is further known that DOS is required for signaling by various other receptors as well. In a recent study, the *C. elegans* homolog of DOS, Soc1, was identified as a docking protein involved in EGL-15 (a fibroblast growth factor receptor homolog) signaling, an important interaction for differentiation processes in the nematode (36). Finally, all three mammalian Gab family members have been shown to be involved in signaling pathways downstream of multiple RTKs and non-RTKs (27).

Gab1 was originally isolated as a Grb2-binding protein which becomes tyrosine phosphorylated following epidermal growth factor or insulin receptor stimulation (14). Gab1 was also independently identified as a substrate of the hepatocyte growth factor receptor c-Met, which transmits signals involved in cell morphogenesis (8, 40). In addition, Gab1 is tyrosine phosphorylated upon stimulation of various other growth factor, cytokine, or antigen receptors (15, 17, 23, 31, 39). In its activated form, Gab1 recruits multiple SH2 domain-containing proteins such as the tyrosine phosphatase SHP2, the p85 subunit of phosphatidylinositol 3-kinase (PI3K), the adaptor proteins SHC and Crk/CrkL, and the phospholipase Cγ (PLC-γ) to form signaling complexes that regulate multiple biological processes. The essential role of Gab1 during mouse development has been demonstrated in *Gab1*-deficient mice, which die between embryonic days E13.5 and E18.5 in utero with developmental defects in the heart, placenta, and skin (18, 35).

Similar to Gab1, Gab2 has been identified as a tyrosine-phosphorylated protein following stimulation of a variety of growth factor, cytokine, and antigen receptors (1, 6, 9, 10, 28, 31). In addition to the constitutive interaction of Gab2 with Grb2, activated Gab2 transiently associates with SHP2, PI3K, SHIP, SHC, and CrkL, thereby regulating intracellular signals originating from cell surface receptors. During signaling by the macrophage colony-stimulating factor (M-CSF) receptor, c-Fms, it has been shown that the interaction of Gab2 with SHP2 is involved in the differentiation signal triggered by M-CSF, which induces macrophage development (26). Furthermore, Gab2-PI3K interaction negatively regulates T-cell receptor

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(TCR) signaling in T lymphocytes (33). Despite these in vitro results, which indicate that Gab2 is important for signal transduction in hematopoietic cells, analysis of *Gab2*-deficient mice revealed no apparent defects in hematopoiesis. Although these mice are overall normal in development, mast cells lacking *Gab2* exhibit decreased responses following stimulation of the high-affinity immunoglobulin E (IgE) receptor FcεRI (11). Independently, Nishida et al. demonstrated that the lack of *Gab2* leads to defective mast cell development in *Gab2*-deficient mice (30). Since the RTK c-Kit has been shown to play a major role in mast cell development, it seems likely that Gab2 acts downstream of c-Kit during this differentiation process.

Gab3 has been shown to become activated following M-CSF, interleukin-3 (IL-3), and Flt3 ligand stimulation in vitro (41), although it is likely that Gab3 also acts downstream of other growth factor, cytokine, or antigen receptors. Similar to Gab1 and Gab2, Gab3 constitutively interacts with Grb2 and after tyrosine phosphorylation is able to bind SHP2 and PI3K (41). In contrast, association of Gab3, but not Gab2, with the monocytic adaptor protein Mona could be observed after M-CSF stimulation (5). Both Gab3 and Mona are induced during M-CSF-triggered macrophage differentiation and are engaged in multimolecular complexes associated with the M-CSF receptor c-Fms (5). Overexpression of Gab3 in the murine myeloid progenitor line FDC-P1, engineered to stably express c-Fms, leads to decreased proliferation and enhanced differentiation into macrophages after M-CSF stimulation (41). Together, these data suggest that Gab3 may play an important role in M-CSF-induced signal transduction leading to macrophage differentiation.

To assess the physiological role of Gab3 we have generated *Gab3*-deficient mice. In this study we show that these mice develop normally and are generally healthy. Using Gab3-specific monoclonal antibodies, a predominant expression of Gab3 was detected in lymphocytes and bone marrow (BM)-derived macrophages. Furthermore, in vitro and in vivo studies revealed that *Gab3*-deficient mice have no detectable defects in hematopoiesis, including macrophage development, and are immunocompetent.

MATERIALS AND METHODS

Construction of the *Gab3* targeting vector and generation of mutant mice. The *Gab3* targeting vector was designed to disrupt the gene at exon 2 and produce a translational fusion between codon 28 of *Gab3* and *Escherichia coli* β-galactosidase (Fig. 1A). A genomic clone containing *Gab3* exon 2 was selected from a C57BL/6 genomic library in Lambda Dash (Stratagene, La Jolla, Calif.) by hybridization with a 445-bp *Eco*RI-*Nco*I fragment of the murine *Gab3* cDNA (41) containing the first 378 nucleotides of the open reading frame. A 7-kb internal *Xba*I fragment, which was derived from a positive clone and which hybridizes to the N-terminal cDNA probe, was subcloned and sequenced. This clone contains *Gab3* exon 2, along with flanking regions of introns 1 and 2. The targeting vector contains a 1-kb 5′ *Gab3* fragment, generated by PCR and designed for in-frame fusion after codon 28, and a 5-kb 3′ *Eco*RI-*Xba*I fragment to facilitate homologous recombination. It contains further a neomycin phosphotransferase (*Neo*) coding sequence driven by a phosphoglycerate kinase promoter for positive selection and a diphtheria toxin gene outside of the homologous recombination cassette for negative selection. The nucleotide sequence of the construct was confirmed prior to electroporation of the linearized vector into 129/Sv.jae murine embryonic stem (ES) cells, which were subsequently grown in the presence of selection medium. Targeted ES cells were injected into C57BL/6 blastocysts, which were subsequently transferred to pseudopregnant mice to generate chimeric offspring. Chimeras were then crossbred with C57BL/6 mice to achieve germ line transmission of the *Gab3* disrupted allele.

PCR and Southern blot screening. Genomic DNA from 1-cm mouse tail clippings was isolated by incubation with agitation for 5 h at 55°C in lysis buffer (100 mM Tris-HCl [pH 8.5], 5 mM EDTA, 0.2% sodium dodecyl sulfate, 200 mM NaCl, 0.1 mg of proteinase K/ml) followed by isopropanol precipitation. Routine genotyping through PCR was performed with the Advantage 2 polymerase kit (Clontech, Palo Alto, Calif.) on 1 μg of genomic DNA, using specific oligonucleotide primers for the *Gab3* wild-type allele (p1, 5′-CTGAACTTTGTCTCT GTACCTC-3′; p2, 5′-ACTAGGATTGCCACTCATGC-3′) and for the targeted allele (p1, described above; p3, 5′-TCCTGTAGCCAGCTTTCATC-3′). PCR conditions for amplification of the wild-type and targeted alleles were 1 cycle at 94°C for 1 min followed by 40 cycles at 94°C for 30 s and 68°C for 2 min.

For Southern blot analysis, genomic DNA was purified from livers of *Gab3*$^{-/-}$ mice or littermate controls and from *Gab3*$^{-/-}$ ES cells by proteinase K digestion and phenol extraction. Ten micrograms of DNA was digested with the restriction enzyme *Pst*I, separated on a 0.7% agarose gel, and blotted to Hybond N membrane (Amersham Pharmacia Biotech, Piscataway, N.J.) in 0.4 N NaOH, 1 M NaCl. Blots were probed with random-primed, ^{32}P-labeled cDNA fragments generated by reverse transcription-PCR (RT-PCR) from either *Gab3* exon 2 or *LacZ* according to standard methods.

Histopathology. The brain, heart, liver, lung, kidney, spleen, and thymus were isolated from 6-week-old *Gab3*$^{-/-}$ and wild-type littermate mice, fixed in 10% buffered formalin, and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin and analyzed by microscopy.

Monoclonal antibody production. Gab3-specific monoclonal antibodies were generated by immunizing BALB/c mice with a glutathione *S*-transferase (GST)-tagged protein, representing amino acids 185 to 595 of murine Gab3. The hybridoma clone P5G9 was selected for its ability to specifically immunoprecipitate Gab3 and detect the denatured protein in Western blot analysis. Monoclonal antibodies were purified by affinity chromatography using protein A-Sepharose columns (Amersham Pharmacia Biotech).

IP and Western blot analysis. Cell suspensions of liver, lung, spleen, thymus, and BM cells, harvested from femurs, were obtained by mincing the organs through sterile 80-μm metal mesh sieves and filtering the suspensions through 40-μm nylon mesh cell strainers. Peripheral blood (PB) was collected by cardiac puncture of C57BL/6 mice. Erythrocytes present in PB, BM, and spleen samples were lysed using 150 mM ammonium chloride buffer. Protein lysates were made by incubating the cell suspensions for 1 h at 4°C in lysis buffer (50 mM Tris-HCl [pH 7.3], 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 5 μM $ZnCl_2$, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin/ml, 2 mM orthovanadate). Lysates were cleared of cell debris by centrifugation, and supernatants were normalized for total protein levels. Immunoprecipitations (IPs) were performed by incubating protein lysates for 1 h with anti-Gab3 polyclonal serum (41) and protein A-Sepharose (Amersham Pharmacia Biotech) (2 μl and 20 μl per mg of total protein, respectively). Following five washes with lysis buffer, precipitated proteins were separated by sodium dodecyl sulfate–4-to-12% gradient polyacrylamide gel electrophoresis (Invitrogen, Carlsbad, Calif.), transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.), and detected with purified P5G9 monoclonal antibody (0.5 μg/ml). Gab3 protein bands were visualized by using horseradish peroxidase-coupled anti-mouse IgG secondary antibody (Jackson ImmunoResearch, West Grove, Pa.) and enhanced chemiluminescence detection (Dupont-NEN, Boston, Mass.).

BM in vitro cultures. To remove stroma cells and monocytes, BM single-cell suspensions from *Gab3*$^{-/-}$ and control mice were incubated for 4 h in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS) in plastic culture flasks. Nonadherent cells were collected and adjusted to 1.5×10^6 cells/ml in DMEM plus 10% FBS plus 1,000 U of M-CSF/ml. One milliliter of the cell suspension per well was seeded into a six-well plate and incubated for 6 days at 37°C, 5% CO_2 in air by exchanging the medium daily. Adherent macrophages were harvested using trypsin-EDTA, and the number of cells was determined using a hemacytometer. Cells were further used for either RNA isolation or to obtain protein lysates.

Magnetic-activated cell sorter and fluorescence-activated cell sorter (FACS) analyses. CD19$^+$ B cells were positively selected from single-cell suspensions of *Gab3*$^{-/-}$ and littermate control BM by labeling the cells with magnetic bead-conjugated antibodies against CD19 (Miltenyi Biotec, Auburn, Calif.), followed by AutoMACS magnetic bead sorting according to the manufacturer's instructions. Similarly, CD4$^+$ T cells, CD8$^+$ T cells, and CD19$^+$ B cells were isolated by positive selection from single-cell suspensions of *Gab3*$^{-/-}$ and littermate control spleens by successively labeling the cells with magnetic bead-conjugated antibodies against CD4, CD8, and CD19 (Miltenyi Biotec), followed by AutoMACS sorting. *Gab3*$^{-/-}$ and wild-type thymocytes were sorted by flow cytometry into mature CD4 single-positive, mature CD8 single-positive, and immature CD4 and CD8 double-positive T lymphocytes by using a Becton Dickinson FACSVantage.

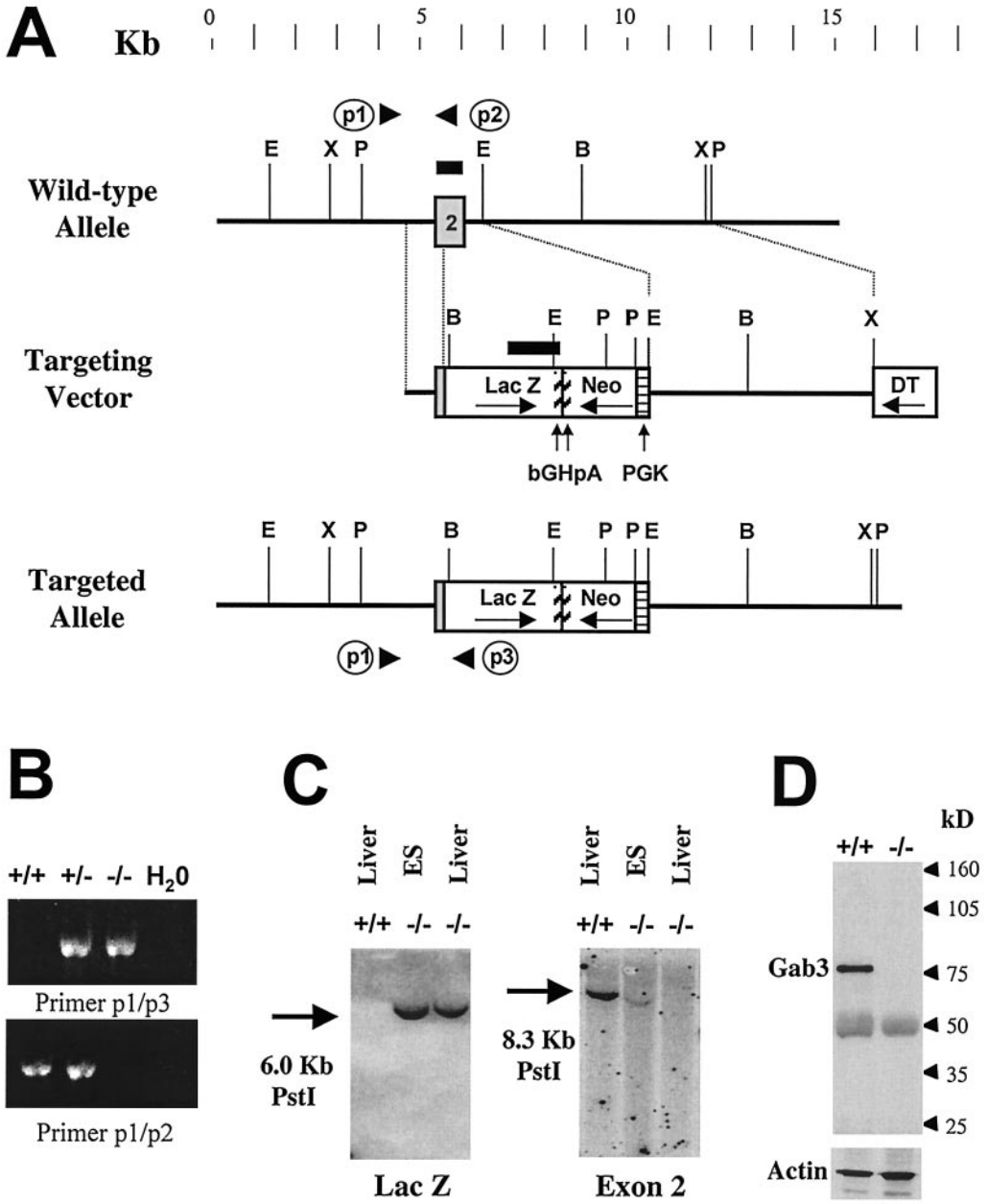

FIG. 1. Targeted disruption of *Gab3* in mice. (A) Schematic representation of the *Gab3* locus, the targeting construct, and the disrupted allele. The shaded box represents total or partial *Gab3* exon 2, and the blank boxes represent the genes for neomycin resistance (*Neo*) for positive selection and diphtheria toxin (DT) for negative selection, as well as *LacZ* for β-galactosidase expression, which replaces expression of Gab3 in the targeted allele. A phosphoglycerate kinase promoter driving *Neo* expression and bovine growth hormone poly(A) addition sites (bGHpA), as well as the positions of the primers p1, p2, and p3 (black arrows), which were used for PCR, and the *LacZ* and *Gab3* exon 2 probes (black bars), which were used for Southern blotting, are indicated. (B) Genotyping of mice for the targeted disruption was performed by PCR using the primer combinations p1-p2, which is specific for the wild-type allele, and p1-p3, which amplifies the targeted allele. (C) Homologous recombination of the targeting vector in mice was verified by Southern blot analysis. Genomic DNA isolated either from wild-type or *Gab3*$^{-/-}$ liver or from targeted ES cells was digested with *Pst*I and hybridized either with a *LacZ*-specific probe recognizing a 6.0-kb band in the targeted allele or with a *Gab3* exon 2-specific probe resulting in an 8.3-kb signal in the wild-type allele. (D) IP experiments with polyclonal anti-Gab3 serum followed by Western blot analysis using Gab3-specific monoclonal antibody confirms the lack of Gab3 protein in various tissues of *Gab3*$^{-/-}$ mice (lanes show thymus samples from wild-type and *Gab3*$^{-/-}$ mice). In the lower portion of the figure, an actin-specific Western blot is shown to confirm equal loading of the two lanes.

Flow cytometry analyses of all sorted cells were performed using fluorochrome-conjugated antibodies to monitor the purity of the populations.

Flow cytometry analysis and automated cell counting. PB from *Gab3*$^{-/-}$ mice and wild-type littermates was collected from the retroorbital vein into EDTA-containing medium. Total and differential white blood cell counts, as well as red blood cell and platelet counts and red blood cell parameters (mean corpuscular volume, red cell distribution width, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and hematocrit) of these samples were an-

alyzed using a Cell-Dyn automated cell counter (Harrell Medical, Inc., Lake Oswego, Oreg.). Single-cell suspensions of BM, spleen, and thymus cells were obtained as described above in "IP and Western blot analysis." Peritoneal cells were isolated by peritoneal lavage using 10 ml of phosphate-buffered saline (PBS) with 2% FBS. Total cell counts of these suspensions were obtained by using a Z2 Coulter Counter (Beckman Coulter, Miami, Fla.). Differential cell counts of thymus, spleen, BM, and peritoneal fluid were acquired by flow cytometry analysis after staining single-cell suspensions using antibodies to the following proteins: fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (clone 17A2), CD4 (L3T4), CD19 (1D3), Ly-6C (AL-21), Ly-6G/Gr-1 (RB6-8C5), CD117/c-Kit (2B8), and pan-NK cells (DX5) (BD Pharmingen, San Diego, Calif.); phycoerythrin-conjugated anti-CD8a (clone 53-6.7), CD45R/B220 (RA3-6B2), CD11b/Mac1 (M1/70), Ly-76 (TER-119), and Sca-1 (D7) (BD Pharmingen); as well as FITC-conjugated anti-F4/80 antibody (Serotec Inc., Raleigh, N.C.). To prevent nonspecific binding of labeled antibodies to Fc receptors on the cells, unlabeled anti-CD16/CD32 antibody (Fcγ III/II R; clone 2.4G2; BD Pharmingen) was added to the cells prior to the addition of labeled reagents. Staining was performed for 30 min on ice in FACS buffer (PBS containing 0.5% bovine serum albumin and 0.1% sodium azide). After washing twice in FACS buffer, cells were immediately acquired on a Becton Dickinson FACSCalibur and analyzed using CellQuest software.

Colony-forming assay. Aliquots of 1.5×10^5 BM cells or 1.5×10^6 splenocytes from *Gab3*$^{-/-}$ or control mice were added to 3 ml of semisolid methylcellulose-based medium (MethoCult GF M3434; StemCell Technologies, Vancouver, British Columbia, Canada) containing stem cell factor, IL-3, IL-6, and erythropoietin. Twice, 1.1 ml of the medium was dispensed into two 35-mm dishes to prepare duplicated colony-forming assays. The number of erythroid burst-forming units (BFU-E), granulocyte/macrophage CFU (CFU-GM), and granulocyte/erythroid/macrophage/megakaryocyte CFU (CFU-GEMM) were counted after 7 and 12 days of incubation at 37°C, 5% CO_2 in air and >95% humidity.

Macrophage activity assay. Peritoneal macrophages of *Gab3*$^{-/-}$ and control mice were isolated by peritoneal lavage using 10 ml of PBS plus 2% FBS. Activation of macrophages was achieved by a two-step procedure using the following concentrations of gamma interferon (IFN-γ). In a round-bottom 96-well plate, 2×10^6 peritoneal cells per well were incubated in 150 μl of DMEM plus 10% FBS and 10 U of IFN-γ/ml for 4 h at 37°C, 5% CO_2 in air to prime macrophages. Triggering of the cells was achieved by increasing the amount of IFN-γ in the samples to a final concentration of 60 U/ml. After 24 and 48 h of incubation at 37°C, 5% CO_2 in air, 50 μl of culture fluid from each sample was collected, and the production of NO_2^- by the activated macrophages was determined by adding 50 μl of 1% sulfanilamide in 2.5% phosphoric acid and 50 μl of 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid to the supernatant and measuring the absorbance of the samples at 550 nm in a microplate reader. Serial dilutions of $NaNO_2$ were used to plot a standard curve and to calculate the amount of NO_2^- in the samples.

RT-PCR. Total RNA from cells or tissues was isolated using a QIAGEN RNeasy kit (QIAGEN Inc., Valencia, Calif.) according to the manufacturer's instructions. Semiquantitative RT-PCR was performed as previously described (41). Briefly, after DNase I digestion of the RNA sample, the first-strand reaction was performed using Superscript II reverse transcriptase (Invitrogen). cDNA was subsequently amplified with *Taq* polymerase using specific oligonucleotide primers for Gab1, Gab2, Gab3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described elsewhere (41). PCR products were quantified on ethidium bromide-stained gels using a Typhoon 8600 scanner (Amersham Biosciences, Sunnyvale, Calif.) and ImageQuant software.

Passive systemic anaphylaxis. Eight-week-old *Gab3*$^{-/-}$ mice and control littermates were sensitized for 24 h with 2 μg of anti-2,4-dinitrophenol (DNP) IgE (Sigma, St. Louis, Mo.) by intravenous injection. Subsequently, the mice were challenged by intravenous injection of 500 μg of DNP-human serum albumin (HSA) (Sigma). After 1.5 min, the mice were euthanized with CO_2, and blood was immediately collected by cardiac puncture. Serum histamine concentrations were determined using a competitive histamine immunoassay kit (Immunotech, Marseille, France).

Delayed-type hypersensitivity reaction. Eight-week-old *Gab3*$^{-/-}$ and littermate controls received a subcutaneous injection of 100 μg of ovalbumin emulsified with an equal volume of complete Freund's adjuvant (OVA-CFA) in the footpad. After 4 weeks the mice received a second subcutaneous OVA-CFA injection in the same footpad, and the thickness of the pad was measured before and 7 days after that second injection by using a thickness-gauge caliper. Serum IgG1 levels from OVA-CFA-treated *Gab3*$^{-/-}$ and control mice were measured before and 4 weeks after the first injection by using a mouse IgG1 enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories, Montgomery, Tex.).

LCMV infection. Six- to 8-week-old *Gab3*$^{-/-}$ and littermate controls were infected with 2×10^5 PFU of the Armstrong strain of lymphocytic choriomeningitis virus (LCMV) by intraperitoneal injection. At the peak of the T-cell response to LCMV (8 days postinfection), splenocytes were harvested and stained with fluorochrome-conjugated anti-CD8, anti-CD4, and anti-CD44 (clone IM7; BD Pharmingen) antibodies to analyze activated populations. To quantitate virus-specific $CD8^+$ T cells, splenocytes were cultured for 5 h at 37°C with the LCMV peptides NP205-212, GP276-286, GP33-41, and NP396-404 (1 μM) in the presence of Brefeldin A (1 μl/ml), and staining for intracellular IFN-γ was done as previously described (29). LCMV-specific IgG in serum from LCMV-infected *Gab3*$^{-/-}$ and control mice was measured by enzyme-linked immunosorbent assay as previously described (37).

RESULTS

Generation of *Gab3*-deficient mice. To assess the physiological function of Gab3, we generated *Gab3*-deficient mice. The targeting vector was constructed by substituting most of *Gab3* exon 2 with a *Neo*- and *LacZ*-containing cassette in order to generate a null mutation (Fig. 1A). Blastocysts, microinjected with ES cells carrying a *Gab3*-targeted allele, were transferred to pseudopregnant mice for production of chimeric offspring. Crossbreeding these chimeras with C57BL/6 mice generated female heterozygous mice carrying one normal and one mutant *Gab3* germ line allele and male offspring which were identified by PCR as hemizygous for *Gab3* deficiency. Subsequent fluorescent in situ hybridization (FISH) analysis revealed that the *Gab3* allele is localized to the X chromosome, explaining the absence of heterozygous male animals (data not shown). A GenBank search revealed a partial *Gab3* sequence at chromosome XB in the mouse and a complete *Gab3* homolog in humans at chromosome Xq28. No obvious phenotype was apparent in heterozygous or hemizygous *Gab3* mutant mice, and matings with these animals produced offspring with the expected Mendelian distribution of wild-type, heterozygous, hemizygous, and homozygous *Gab3* mutant animals (data not shown), which were genotyped by PCR and Southern blot analysis (Fig. 1B and C).

To confirm that mice hemizygous or homozygous for the targeted mutation of *Gab3* (referred to as *Gab3*$^{-/-}$ mice) do not express Gab3 protein, IP followed by Western blot analysis using Gab3-specific antibodies was performed with various tissues from wild-type and mutant mice. A band for Gab3 protein of the expected size could be clearly detected in wild-type animals, but not in *Gab3*$^{-/-}$ mice (Fig. 1D). In addition, no truncated or spliced isoforms of Gab3 protein were detected with either a polyclonal or monoclonal Gab3-specific antibody (see next section) in tissues of *Gab3*$^{-/-}$ mice. The lack of Gab3 expression was confirmed by RT-PCR analysis using *Gab3*-specific primers (data not shown). In addition, we confirmed the expression and activity of β-galactosidase in *Gab3*$^{-/-}$ mice by RT-PCR analysis using *LacZ*-specific oligonucleotide primers, as well as by FACS-Gal measurements using splenocytes isolated from *Gab3*-deficient animals (data not shown).

Mice lacking *Gab3* and age-matched wild-type littermates were indistinguishable in appearance and average weight, and no behavioral changes were seen when handling *Gab3*$^{-/-}$ mice compared to wild-type animals. Furthermore, there was no significant difference in mortality between mutant and control mice up to 12 months of age. A histopathological analysis of adult brain, heart, liver, lung, kidney, spleen, and thymus from *Gab3*$^{-/-}$ animals revealed grossly normal morphologies in all

tissues (data not shown). These data indicate that *Gab3* is not essential for normal mouse development.

Expression of *Gab3*. To examine the expression pattern of *Gab3* at the protein level, we raised Gab3-specific monoclonal antibodies by immunizing BALB/c mice with a Gab3-GST fusion protein as described in Materials and Methods. In contrast to the previously described polyclonal rabbit anti-mouse Gab3 antiserum (41), which cross-reacts with Gab2 (unpublished observation), we generated a highly Gab3-specific monoclonal antibody, termed clone P5G9. Western blot analysis with P5G9 revealed that Gab3 is generally expressed at very weak levels, as we were not able to detect the protein in any of the analyzed tissue lysates directly. Therefore, IP experiments followed by Western blotting were used to analyze protein expression in tissues. By this means we detected the strongest expression of Gab3 protein in mouse spleen and thymus and weaker expression in PB, BM, lung, and liver lysates from wild-type mice (Fig. 2A). No Gab3 protein could be detected in brain, heart, or kidney samples (data not shown). These results confirm the previously described RT-PCR data, which showed the strongest expression of Gab3 in spleen, thymus, and hematopoietic cell lines (41).

Despite the low expression of Gab3 in primary BM cells, a dramatic increase in Gab3 protein levels was observed after differentiation of these cells into macrophages, which was induced by incubation of BM cells for 6 days in M-CSF-containing medium (Fig. 2B). Similarly, we observed increased expression of Gab3 protein after culturing BM cells for 6 weeks in the presence of IL-3 (data not shown). Flow cytometry analysis confirmed that the main cell type in these cultures was mast cells, expressing the high-affinity IgE receptor, FcεRI.

To identify Gab3-expressing cell subsets within the BM, spleen, and thymus, we performed cell sorting experiments using antibodies against the lymphocyte-specific cell surface markers CD4, CD8, and CD19. Subsequent FACS analysis revealed a purity of >95% of the sorted cell populations. IP and Western blot analysis, using protein lysates from these populations, demonstrated that Gab3 protein was expressed in BM-derived CD19$^+$ B lymphocytes but not in the CD19-depleted BM cells, representing mainly cells of the myeloid lineage (Fig. 2C). Strong expression of Gab3 was further detected in CD8$^+$ T cytotoxic cells, CD4$^+$ T helper cells, and CD19$^+$ B lymphocytes of the spleen (Fig. 2C). Within the thymus we detected expression of Gab3 in the CD4$^+$ CD8$^+$ immature thymocyte population, as well as a weaker expression in mature single-positive CD4 or CD8 T lymphocytes (Fig. 2C).

Together, these data demonstrate that Gab3 protein is primarily expressed in hematopoietic cells, particularly those of lymphoid lineage. Further, Gab3 expression is increased during myeloid cell differentiation.

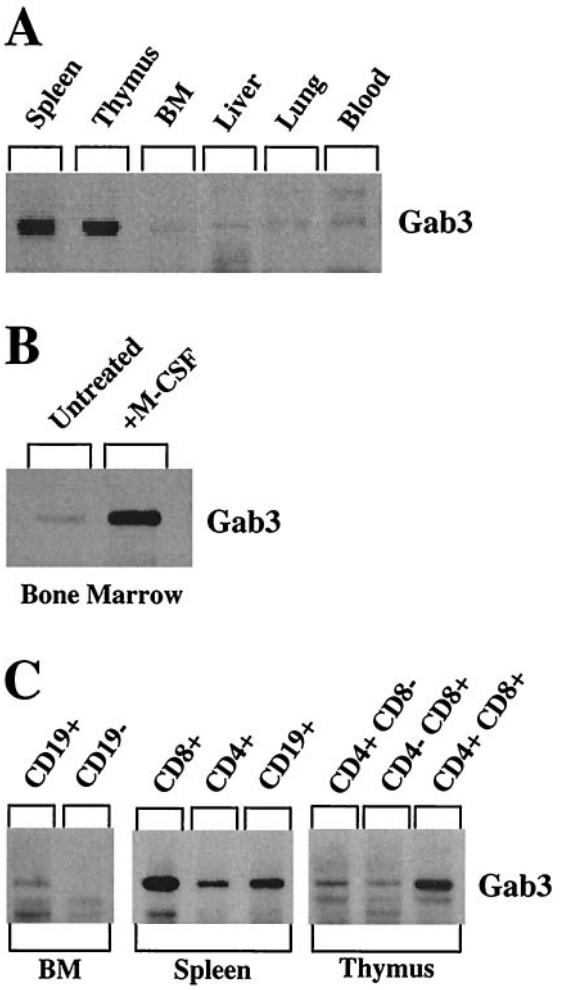

FIG. 2. Expression of Gab3 protein. (A) Gab3 was immunoprecipitated using polyclonal anti-Gab3 serum from protein lysates of the indicated tissues of mice and detected by Western blot analysis using the Gab3-specific monoclonal antibody P5G9. (B) Primary BM cells isolated from mice were differentiated into macrophages by culturing them for 6 days in M-CSF. IP and Western blot analysis was done as described in the legend for panel A by using normalized protein lysates of cells before and after M-CSF treatment. (C) A single-cell suspension of C57BL/6 BM was separated by magnetic bead-conjugated antibodies and AutoMACS into CD19$^+$ B lymphocytes and CD19$^-$ cells. The cell suspension of spleen cells was separated into CD4$^+$ T-cell, CD8$^+$ T-cell, and CD19$^+$ B-cell subpopulations by AutoMACS. Thymocytes were separated into CD4$^+$ CD8$^-$, CD4$^-$ CD8$^+$, and CD4$^+$ CD8$^+$ subpopulations by flow cytometry. Expression of Gab3 in all these cell populations was analyzed by IP and Western blot analysis as described above, using Gab3-specific antibodies.

Hematopoiesis in *Gab3*$^{-/-}$ mice. To determine if lack of *Gab3* expression affected development of hematopoietic cells, a thorough analysis of the hematopoietic compartment in *Gab3*$^{-/-}$ mice was performed. Total and differential cell counts of PB, BM, thymus, spleen, and peritoneal fluid were obtained through automated cell counting and flow cytometry analysis using cell surface markers for T and B cells, erythroid cells, NK cells, monocytes, macrophages, granulocytes, and hematopoietic progenitor cells. Red blood cell parameters (e.g., hematocrit) and platelet counts of total blood samples were comparable between *Gab3*$^{-/-}$ and wild-type littermates (data not shown). In addition, all leukocyte populations were present at normal frequencies (Table 1). To quantitate hematopoietic progenitor cells in the BM and spleens of *Gab3*-deficient mice and wild-type littermates, we performed colony-forming assays using methylcellulose-based media supplemented with growth factors to support the growth of erythroid (BFU-E), myeloid (CFU-GM), and mixed-lineage colonies (CFU-GEMM). We observed no significant differences in the numbers of hematopoietic progenitors from *Gab3*$^{-/-}$ animals versus controls by

TABLE 1. Flow cytometry analysis of hematopoietic subpopulations

Cell type	Control (%)[a]	*Gab3*$^{-/-}$ (%)[a]
Bone marrow		
CD4$^+$ T cells	0.7 ± 0.1	0.9 ± 0.0
CD8$^+$ T cells	0.9 ± 0.2	1.4 ± 0.5
B220$^+$ B cells	20.3 ± 3.4	21.7 ± 1.2
Ter119$^+$ erythroid cells	2.6 ± 1.1	3.0 ± 0.4
Mac1$^+$ DX5$^+$ NK cells	3.4 ± 0.2	3.2 ± 1.0
Mac1$^+$ Ly6C^{high} monocytes	11.8 ± 1.8	12.0 ± 2.2
Gr1$^+$ Ly6C^{low} DX5$^-$ neutrophils	61.8 ± 6.0	56.2 ± 0.7
Sca1$^+$ c-Kit$^+$ progenitor cells	2.6 ± 0.6	2.4 ± 0.5
Spleen		
CD4$^+$ T cells	15.4 ± 1.2	16.0 ± 1.6
CD8$^+$ T cells	9.3 ± 1.0	10.6 ± 1.9
B220$^+$ B cells	62.4 ± 2.2	60.4 ± 3.5
Ter119$^+$ erythroid cells	6.4 ± 2.9	6.3 ± 0.9
Mac1$^+$ DX5$^+$ NK cells	3.5 ± 0.6	4.7 ± 1.0
Mac1$^+$ F4/80$^+$ macrophages	4.8 ± 1.4	5.9 ± 1.9
Thymus		
CD4$^-$ CD8$^-$	4.9 ± 2.4	3.7 ± 0.5
CD4$^+$ CD8$^-$	9.5 ± 3.5	8.5 ± 4.1
CD4$^-$ CD8$^+$	3.0 ± 1.1	2.9 ± 1.3
CD4$^+$ CD8$^+$	82.6 ± 4.9	85.0 ± 5.5
Peritoneum		
Mac1$^+$ F4/80$^+$ macrophages	50.1 ± 7.3	50.0 ± 6.2

[a] Percentages of cell subsets in *Gab3*$^{-/-}$ mice and control littermates are shown. Values represent means ± standard deviations of three separate determinations.

counting hematopoietic colonies after 7 and 12 days of culture (data not shown). These data suggest that *Gab3* is not essential for the development of hematopoietic cell lineages.

Macrophage differentiation and activation in the absence of *Gab3*. Previous in vitro studies with the murine myeloid progenitor cell line FDC-P1 engineered to express exogenous M-CSF receptor (FD-Fms cells) indicated that overexpression of Gab3 in these cells accelerated macrophage differentiation triggered by M-CSF (41). To determine whether Gab3 is important for primary macrophage development, we cultured BM cells from *Gab3*$^{-/-}$ mice and age-matched wild-type littermates for 6 days in the presence of M-CSF and then counted the number of resulting adherent macrophages in culture. We observed no significant differences in the number of recovered macrophages between *Gab3*$^{-/-}$ and control animals ([5.1 ± 1.2] × 10^5 cells for *Gab3*$^{-/-}$ mice versus [5.8 ± 0.6] × 10^5 cells for wild-type mice; $n = 5$).

One possible explanation for the observed differences between FD-Fms cells and primary BM cells could be that other Gab family members compensated for the lack of *Gab3* during macrophage development. To assess such a compensating effect, we performed semiquantitative RT-PCR analysis to measure Gab1 and Gab2 mRNA levels in *Gab3*$^{-/-}$ and littermate control BM cells cultured in M-CSF-containing medium. Consistent with previous results, Gab1 and Gab2 expression was up-regulated during macrophage differentiation (41). However, we observed no significant differences in Gab1 and Gab2 mRNA levels between *Gab3*-deficient or control cells during this development (Fig. 3).

We further analyzed the cytotoxic potential of immunologically stimulated macrophages isolated from the peritoneal cavity of *Gab3*$^{-/-}$ mice or wild-type littermates by assaying the production of reactive nitrite in vitro. No significant difference in macrophage activity was detected when comparing *Gab3*-deficient and control animals (data not shown).

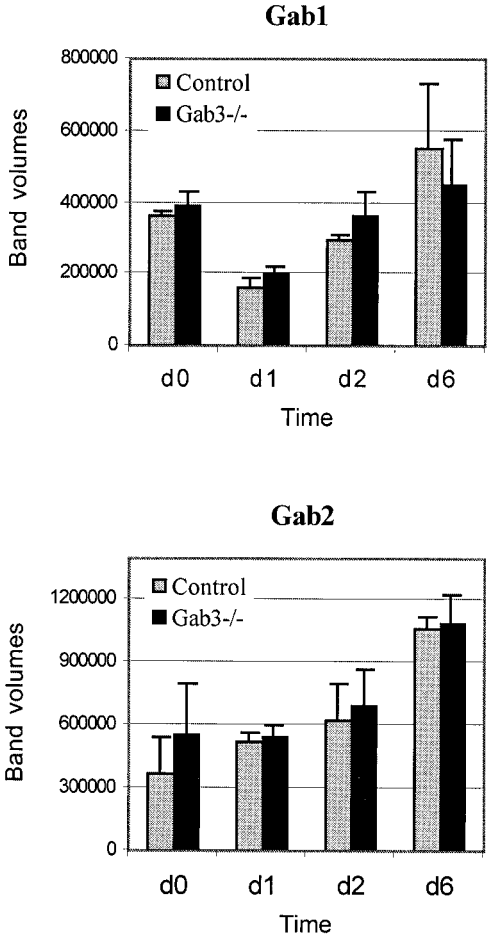

FIG. 3. Expression levels of Gab1 and Gab2 during macrophage differentiation in *Gab3*$^{-/-}$ and control littermates. BM cells isolated from *Gab3*$^{-/-}$ and control mice were cultured for 6 days in M-CSF. Total RNA was isolated from cells prior to the addition of M-CSF and at the indicated time points. Gab1 and Gab2 mRNA levels were quantitated by RT-PCR analysis using Gab1- or Gab2-specific primers. Quantification of ethidium bromide-stained DNA bands in gels was performed with ImageQuant software. Gab1 and Gab2 signals were normalized to RT-PCRs using GAPDH-specific primers. Results and standard deviations of five independent experiments are shown.

Together, these data show that *Gab3* is not essential for the development of BM-derived, primary macrophages or the function of peritoneal macrophages, as measured by the production of reactive nitrite. Further, the lack of *Gab3* during macrophage differentiation is not compensated for by an increased expression of Gab1 or Gab2.

Immune responses in *Gab3*$^{-/-}$ mice. Since Gab3 is expressed in cells of the lymphoid lineage, in macrophages, and in mast cells, we next investigated the involvement of Gab3 during adaptive immune responses. It has been recently shown that *Gab2*$^{-/-}$ mice exhibit a severe defect in allergic responses, due to defective signaling through the high-affinity IgE receptor, FcεRI, in the absence of *Gab2* (11). To test whether *Gab3* might also play a role in allergic responses, we induced passive

TABLE 2. Immune responses in *Gab3*$^{-/-}$ and control mice

Type of response	Mean response[e]	
	Control	*Gab3*$^{-/-}$
Allergic response[a]	36.6 ± 9.8	44.9 ± 3.8
Delayed-type hypersensitivity[b]	0.93 ± 0.21	0.73 ± 0.06
B-cell response to ovalbumin[c]	688 ± 152	679 ± 119
B-cell response to LCMV[d]	58.3 ± 14.9	66.8 ± 14.8

[a] Histamine serum levels in micromolar after induction of passive systemic anaphylaxis.
[b] Footpad swelling in millimeters 7 days after second subcutaneous ovalbumin injection.
[c] Increase in IgG1 serum levels in micrograms per milliliter 4 weeks after first subcutaneous ovalbumin injection.
[d] Reciprocal dilution of mouse serum 30 days after LCMV infection which gives half maximal response.
[e] Values represent the mean ± standard deviation of three separate determinations.

systemic anaphylaxis in *Gab3*$^{-/-}$ animals and wild-type littermates by sensitizing the mice with anti-DNP IgE and 24 h later challenging them with DNP-HSA. Histamine levels in the serum of *Gab3*$^{-/-}$ and littermate controls in response to this treatment were similar, as determined by competitive histamine immunoassay (Table 2). We further detected no difference in the development of mast cells in vitro, as described previously for *Gab2*-deficient cells (30), by culturing primary BM cells of *Gab3*$^{-/-}$ mice and control littermates for 6 weeks in the presence of IL-3 (data not shown).

We next assessed the responsiveness of *Gab3*-deficient CD4$^+$ T cells by measuring footpad swelling in *Gab3*$^{-/-}$ and control littermates following footpad challenge with OVA-CFA. This delayed-type hypersensitivity reaction has previously been shown to be dependent on skin-localized macrophages and adequate CD4$^+$ T-cell help, and the degree of swelling is an indication of the magnitude of the CD4$^+$ T-cell response (32). We observed similar levels of footpad swelling between the two groups of mice (Table 2), suggesting that *Gab3*-deficient CD4$^+$ T cells are competent in vivo.

To assess the ability of *Gab3*-deficient CD8$^+$ T cells to respond to antigen, we challenged *Gab3*$^{-/-}$ and control littermates with LCMV. Both groups of mice were able to control LCMV infection by 8 days postinfection (data not shown). In addition, we observed a similar profound expansion in CD8$^+$ T cells in both *Gab3*$^{-/-}$ and control mice which resulted in an inversion in the splenic CD4/CD8 ratio compared to the ratio in uninfected mice (Fig. 4A). Since this change in T-cell ratio is indicative of strong CD8$^+$ T-cell activation and expansion, we next used the activation marker CD44 to determine the absolute numbers of activated T cells in LCMV-infected *Gab3*$^{-/-}$ and control littermates. We observed an ~200-fold increase in CD44^{high} CD8$^+$ T cells and a 5- to 10-fold increase in CD44^{high} CD4$^+$ T cells in splenocytes from *Gab3*$^{-/-}$ and littermate control mice, indicating that a similar activation and expansion of CD8$^+$ and CD4$^+$ T cells occurred in *Gab3*$^{-/-}$ and control mice in response to LCMV infection (Fig. 4B and C). We next quantitated LCMV-specific CD8$^+$ T cells by intracellular cytokine staining for IFN-γ following in vitro restimulation with defined LCMV peptide epitopes. We observed similar numbers of LCMV-specific CD8$^+$ T cells in LCMV-infected *Gab3*$^{-/-}$ and littermate controls (Fig. 4D). Furthermore, we observed a similar hierarchy between *Gab3*$^{-/-}$ and littermate control mice in CD8$^+$ T cells specific for LCMV epitopes, strongly suggesting that *Gab3*$^{-/-}$ mice are able to mount normal T-cell responses to LCMV (Fig. 4E).

To determine if B-cell responses were also similar in *Gab3*-deficient mice, we measured serum antibody levels in *Gab3*$^{-/-}$ and littermate control mice immunized with OVA-CFA or LCMV. Since IgG responses to both these immunogens are dependent on CD4$^+$ T-cell help, this also gives an indication of the effectiveness of CD4$^+$ T-cell responses in *Gab3*$^{-/-}$ mice. We observed similar levels of total IgG1 after OVA-CFA immunization in *Gab3*$^{-/-}$ and control littermates as well as similar levels of LCMV-specific IgG in both groups of mice (Table 2).

Taken together, these data show that the lack of Gab3 does not result in a major defect in the development, maturation, or function of mast cells, CD4$^+$ T cells, CD8$^+$ T cells, or B cells in vivo.

DISCUSSION

Gab family members have been shown to be crucial for signaling pathways leading to differentiation processes in various cell types and organisms. Upon activation, phosphorylated Gab proteins associate with SHP2, the p85 subunit of PI3K, and Crk family proteins and subsequently activate mitogen-activated protein kinase, PI3K, and JNK pathways (27). Gab1 is the most ubiquitously expressed mammalian Gab family member, with a broad expression pattern in embryonic and adult tissues of the mouse. Its essential role during murine development has been demonstrated by the embryonic lethality of *Gab1*-deficient mice (18, 35). Recently it has been shown, however, that Gab1 is dispensable for normal hematopoiesis (19). Transplantation of fetal liver cells from *Gab1*$^{-/-}$ embryos into lethally irradiated mice resulted in only minor abnormalities in leukocyte frequencies in the chimeric animals but identified Gab1 as a negative regulator of thymus-independent antigen type 2 responses triggered by marginal-zone B cells of the spleen.

Gab2 protein is quite abundant in brain, kidney, uterus, testis, and hematopoietic cells, and its functional importance in IL-2, IL-3, and M-CSF signaling has been well established in tissue culture systems (10, 26). Further, in vitro studies have described a role for Gab2 during B-cell and TCR signaling (21, 31, 33, 42). Nevertheless, *Gab2*$^{-/-}$ mice lack an apparent developmental phenotype or obvious defects in hematopoiesis or lymphocyte-mediated immune responses (11, 30).

Gab3 is the least ubiquitously expressed member of the Gab family, with a predominant but generally weak expression in hematopoietic cells (references 5 and 41 and present report). In vitro studies have shown that Gab3 is involved in M-CSF-induced and c-Fms-associated signaling complexes important for macrophage differentiation (5, 41). We have evaluated the in vivo role of Gab3 by generating *Gab3*-deficient mice. The *Gab3* targeting vector was constructed to replace most of *Gab3* exon 2 by a *LacZ*- and *Neo*-containing cassette. Therefore, a potential cryptic promoter within the first intron of *Gab3* was disrupted by homologous recombination in *Gab3*$^{-/-}$ mice. Truncated forms of Gab1 and Gab2 proteins, missing most of the N-terminal pleckstrin homology domain, presumably

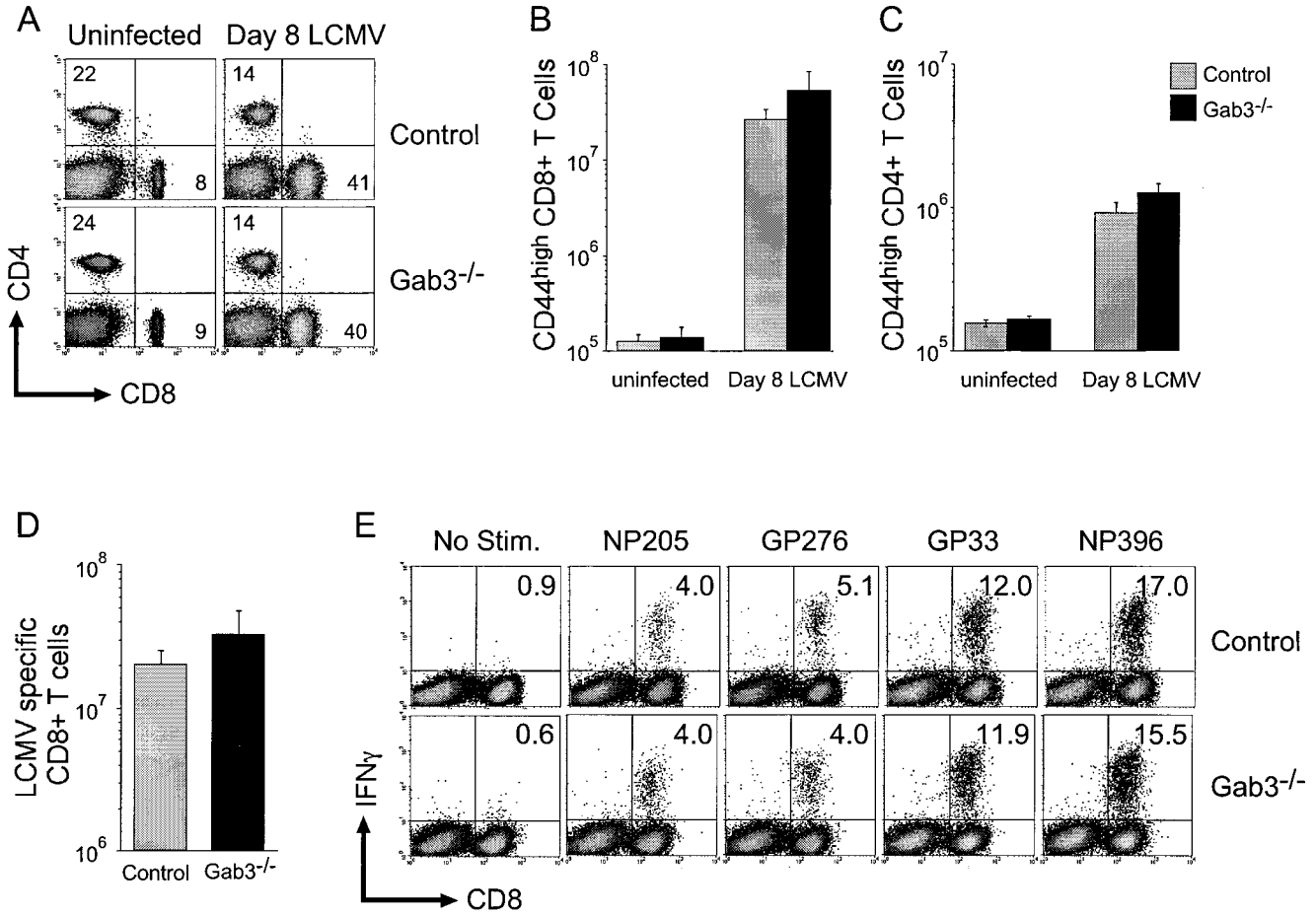

FIG. 4. *Gab3*$^{-/-}$ and control CD8$^+$ and CD4$^+$ T-lymphocyte responses during LCMV infection. (A) Splenocytes from uninfected or LCMV-infected (8 days postinfection) *Gab3*$^{-/-}$ mice and control littermates were stained with anti-CD8, anti-CD4, and anti-CD44 antibodies and analyzed by flow cytometry. Density plots are gated on lymphocytes and percentages indicate the fraction of lymphocytes in each quadrant. (B and C) Numbers of activated CD44^{high} CD8$^+$ (B) and CD44^{high} CD4$^+$ (C) splenocytes from *Gab3*$^{-/-}$ and control LCMV-infected mice at 8 days postinfection compared to numbers in uninfected mice. Gray bars represent control mice; black bars indicate *Gab3*$^{-/-}$ mice. (D and E) Total LCMV-specific CD8$^+$ T cells (D) and their relative immunodominant hierarchy (E) as determined by intracellular staining for IFN-γ following in vitro peptide restimulation. Density plots are gated on lymphocytes, and percentages indicate the fraction of CD8$^+$ T cells specific for each epitope.

driven by such an intrinsic promoter, have been identified in carcinogen-treated Syrian hamster embryo cells (20) and in *Gab2*$^{-/-}$ mice (11), respectively. IP and Western blot analysis with Gab3-specific monoclonal and polyclonal antibodies confirmed the lack of expression of both full-length and truncated Gab3 protein in the *Gab3*$^{-/-}$ mice. However, the lack of Gab3 does not result in major morphological or pathological defects during embryonic development or in the adult mouse. Importantly, even though we detected the strongest expression of Gab3 in hematopoietic cells, we observed no major abnormalities in hematopoiesis or macrophage development in the absence of Gab3. Expression analysis using the murine myeloid progenitor cell line FD-Fms UW or primary BM cells cultured in M-CSF revealed that Gab3 expression is induced during macrophage development (references 5 and 41 and present report). This indicates that Gab3 may be an important mediator of macrophage functions. However, we observed no difference in peritoneal macrophage activity as measured by the production of reactive nitrite in vitro when comparing *Gab3*$^{-/-}$ mice and control littermates. Further, in vitro and in vivo experiments, including induction of passive systemic anaphylaxis, delayed-type hypersensitivity reaction, and LCMV infection revealed that Gab3 is dispensable for the development, maturation, and function of mast cells, CD4$^+$ T cells, CD8$^+$ T cells, or B cells. Nevertheless, we cannot exclude a possible role for Gab3 within more-specialized functions of the immune system. Both LCMV and ovalbumin treatment induce B-cell antibody responses in mice that are highly dependent on CD4$^+$ T-cell help. Since knockout studies have shown that Gab1 as well as the intracellular phosphatase SHIP, which has been shown to interact with Gab2 (26), are involved in thymus-independent B-cell responses (12, 19), a possible involvement of *Gab3* during immune responses after thymus-independent antigen challenges is currently under investigation.

It has been suggested that proteins which are conserved throughout different species are essential for the development and survival of an organism, whereas vertebrate proteins without an ortholog in *Drosophila* or in *C. elegans* may have func-

tions in cells or organs that are more highly evolved in vertebrates, such as the immune system, the cardiovascular system, or the brain (16). A common observation is also that a *Drosophila* gene, like DOS, is represented by a whole family of homologous genes in mammals, like the Gab proteins. In this respect one can speculate that Gab1, with its ubiquitous expression pattern and essential function during embryonic development, may represent *Drosophila* DOS and *C. elegans* Soc1. Whereas, the possibly later-evolved genes *Gab2* and *Gab3* may have more-specialized functions within later-evolved cells or organs like the cells of the immune system, which express Gab2 and Gab3, or the brain, which shows a strong expression of Gab2.

Publications within the last decade have often shown that gene-function relations first established by in vitro studies were not reproducible in knockout mice. One possible explanation for this discrepancy is that other molecules are able to functionally compensate for the lack of a specific gene. Such a compensating effect has been described, for example, for *Src* family members. Mice lacking multiple *Src* family members exhibit more severe and complex phenotypes than mice lacking a single family member (38). Although we observed no differences in the mRNA levels of *Gab1* and *Gab2* in developing macrophages from *Gab3*$^{-/-}$ mice and littermate controls by semiquantitative RT-PCR analysis, it is still possible that Gab proteins have a redundant function in the development or activity of these cells. Even though both Gab1 and Gab2 are up-regulated in developing primary macrophages (41), it seems more likely that Gab2 compensates for the lack of Gab3 in these cells, since Gab1 is not expressed in FD-Fms UW cells differentiating into macrophages (41) and a role for Gab2 during this process has already been described (26). Generation of mice deficient in both *Gab2* and *Gab3* might elucidate a possible redundancy of these two scaffolding proteins and reveal their function in cells of hematopoietic origin.

Double-knockout studies including signaling molecules, which are known to interact with Gab3, might be further helpful to learn more about the function of Gab3 in vivo. One interesting candidate is the adaptor protein Mona/Gads, which is specifically expressed in hematopoietic cells (2, 4, 22, 25) and is up-regulated during macrophage development in a similar way as Gab3 (5). Mona/Gads protein is implicated in TCR signaling (24), and knockout studies showed that thymocyte development is impaired in the absence of this adaptor molecule (43). Crossing of *Gab3*$^{-/-}$ mice with mice deficient for Mona/Gads to produce double knockouts might result in a more severe phenotype within T-cell or macrophage development and function. An extended phenotype is also likely for mice lacking either M-CSF or the M-CSF receptor, c-Fms, in addition to Gab3.

Since in vitro experiments showed that overexpression of Gab3 in FD-Fms UW cells facilitates macrophage differentiation, transgenic animals overexpressing this signaling molecule in hematopoietic cells might show a phenotype in the development or activity of macrophages and therefore help to elucidate the role of Gab3 in vivo.

The described discrepancy between in vitro and in vivo results concerning the biological role of Gab3 might be further explained by the complexity of signaling pathways. Since every single cell coexpresses a variety of receptors and related signaling molecules at various levels or times, the specific outcome, leading to growth, death, or differentiation of the cell, is determined by many different factors. Depending on the combination and level of expression of other signaling molecules, Gab proteins might have minor or major roles downstream of the receptors, resulting in minor or major phenotypes in mice lacking Gab proteins. Since Gab3 protein is expressed generally at very low levels, lack of Gab3 might result in a minor phenotype, which can be detected only by more detailed analysis. To elucidate possible roles of Gab3 in vivo, it is important to identify further signaling pathways which require or result in activation of Gab3. Since both Gab1 and Gab2 are phosphorylated after stimulation of a variety of growth factor, cytokine, or antigen receptors (27), it is very likely that the same is true for Gab3.

Another explanation for the absence of an obvious phenotype in *Gab3*-deficient mice is that Gab3 might play a role in processes which are not critically affecting either growth or development of the animal, such as stress response, DNA repair, or neurological processes resulting in behavioral disorders. Extensive studies using various challenges of the *Gab3*$^{-/-}$ mice might elucidate such a hidden function of Gab3.

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