Unimpaired Macrophage Differentiation and Activation in Mice Lacking the Zinc Finger Transcription Factor NGFI-A (EGR1)

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The zinc finger protein NGFI-A (also called EGR1, Krox24, or zif268) is a candidate regulator of myeloid cell differentiation. Evidence supporting this hypothesis is twofold. First, NGFI-A antisense oligonucleotides prevent macrophage differentiation in HL-60 and U937 myeloid leukemia cell lines and in normal bone marrow cells. Second, enforced expression of NGFI-A blocks granulocytic differentiation and promotes macrophage differentiation in HL-60 cells and in the hematopoietic progenitor cell line 32D. We sought to determine the effect of NGFI-A deficiency on macrophage differentiation and function in vivo by examining native bone marrow cells from mice homozygous for a disrupted allele of NGFI-A derived from gene-targeted ES cells. Macrophages were observed in peripheral blood and several tissues, indicating that NGFI-A was not required for the formation of a variety of macrophage compartments. No differences in myeloid cell differentiation were observed between wild-type and NGFI-A^{-/-} bone marrow cells cultured in the presence of macrophage, granulocyte-macrophage, or granulocyte colony-stimulating factor (M-CSF, GM-CSF, or G-CSF). Activation of NGFI-A^{-/-} macrophages was comparable to that of wild-type macrophages as determined by nitric oxide production and increased cell surface expression of class II major histocompatibility complex molecules. Moreover, NGFI-A^{-/-} mice showed no increased mortality or bacterial burden when challenged with *Listeria monocytogenes*. Together, these results indicate that NGFI-A is not required for macrophage differentiation or activation.

Tissue macrophages and blood monocytes are formed by hematopoiesis, a process by which terminally differentiated blood elements of various lineages arise from multipotential progenitor stem cells (14). This highly regulated process requires exquisite control, mediated in part by colony-stimulating factors (CSFs) such as granulocyte CSF (G-CSF) and macrophage CSF (M-CSF), which act on stem cells at various stages of lineage commitment to promote either granulocytic or monocytic differentiation. Progression through the monocytic lineage involves cell division and maturation through the monoblast, promonocyte, and, finally, monocyte stages. These monocytes then enter and remain in the circulation for several hours before extravasating to tissue spaces to become differentiated macrophages.

When challenged by neoplastic growth, microbial infection, or other pathogenic conditions, macrophages acquire specialized functions through a process classically referred to as activation (24). For example, in the infection model for the facultative intracellular pathogen *Listeria monocytogenes*, macrophages play a central role as the principal effector cells mediating host resistance. Upon encounter with this pathogen, macrophages become activated through a T-cell-independent mechanism to initiate the production of cytokines including interleukin-1, tumor necrosis factor alpha, and interleukin-12 (20). These cytokines initiate multiple responses, including the stimulation of natural killer (NK) cells to release gamma interferon (IFN- γ), which in turn stimulates macrophages to undergo further activation as characterized by the induced expression of various gene products, including nitric oxide synthase, class II major histocompatibility (MHC) molecules, and cytokines. The expression of these molecules endows the mac-

rophages with enhanced bactericidal activity, antigen presentation, and stimulation of cell-mediated immunity.

The genetic programs involved in the progression from one developmental stage to another or the transition from resting to activated macrophages are regulated primarily at the transcriptional level (reviewed in reference 13). Experiments with the myeloblastic leukemia cell line HL-60 and the myelomonocytic cell line U937 have identified several transcription factors whose altered expression correlates with the induction of monocytic differentiation and macrophage activation. These constitute potential regulators of genetic programs and include c-myc, c-myb, c-fos, c-jun, JunB, chx1, PU-1, NF-IL6, IRF-1, IRF-2, Stat-1, Krox-20, and NGFI-A (13).

The importance of NGFI-A in monocytic differentiation was demonstrated in experiments that showed that antisense oligonucleotides to NGFI-A could inhibit macrophage but not granulocytic differentiation (17). This effect was not unique to HL-60 cells, because this observation was extended and confirmed in normal bone marrow cells treated with M-CSF and G-CSF. Furthermore, overexpression of NGFI-A in HL-60 cells restricted differentiation toward macrophages. In more recent experiments with the hematopoietic cell line 32D, which normally does not differentiate into macrophages, further support for this idea was provided by the finding that enforced expression of NGFI-A could surmount the block in differentiation to give rise to macrophages (11). The role of NGFI-A in macrophages may also extend to their activation, because inducers of macrophage activation such as lipopolysaccharide (3) and granulocyte-macrophage CSF (GM-CSF) (21, 25) have been shown to induce NGFI-A.

NGFI-A (also called zif/268, EGR-1, or Krox-24) encodes a transcription factor that was discovered by virtue of its rapid induction during nerve growth factor-induced differentiation (15). Further studies extended its role to a variety of cell types

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FIG. 1. RT-PCR analysis of RNA from peritoneal exudate cells (10^6 cells) induced for 1 h with LPS and IFN- γ was performed with forward and reverse primer sets corresponding to NGFI-A (denoted in the diagram above [not drawn to scale]) or to β -actin. Primer set 1/2 spans the single intron of the NGFI-A gene and would detect potential aberrant splice products that join the open reading frame in exon 1 to the open reading frame in exon 2 that encodes a portion of the DNA-binding domain (ZnF). Primer set 3/4 spans the *neo* insertion and would detect aberrant splice products that excise the *neo* insertion (containing several stop codons in all reading frames), thus joining the 5' half to the 3' half of the second exon. Arrowheads denote the PCR product of the predicted size. The asterisk (*) denotes a nonspecific product present in all lanes including the water blank control (con), which contains only primers and no template. Molecular size markers (in kilobases) are indicated on the right.

induced by a variety of growth, differentiation, and apoptotic stimuli (reviewed in reference 5). NGFI-A is the prototypic member of an immediate-early gene family whose members share a highly homologous C_2H_2 zinc finger domain that confers recognition of a canonical nonamer GCG(T/G)GGGCG (2, 16, 19, 22). In an effort to identify *in vivo* functions that require NGFI-A, we have created mice bearing a targeted disruption of NGFI-A (12). In this report, we analyze macrophage differentiation and function in these mice. In contrast to previous studies, in which antisense methods were used, we found that NGFI-A was not required for macrophage differentiation. Furthermore, macrophages lacking NGFI-A were functional, as assessed by production of nitric oxide (NO) and expression of class II MHC molecules. Finally, we demonstrate that NGFI-A $^{-/-}$ mice possess resistance to *L. monocytogenes* infection.

MATERIALS AND METHODS

NGFI-A mutant mice. Wild-type and homozygous mutant NGFI-A mice were obtained by crossing heterozygous mutant mice bearing a targeted mutation of the NGFI-A gene. Genotypes were determined by Southern blot analysis as previously described (12). Mice were maintained in a C57BL/6- and -129Sv hybrid background and were age (8 to 12 weeks) and sex matched in each experiment. The animals were housed and bred in a specific-pathogen-free facility with an 07:00 to 19:00 light-dark cycle. Procedures were conducted in accordance with guidelines established by the Animal Studies Committee of Washington University and the National Institutes of Health.

RT-PCR analysis. Peritoneal exudate cells (10^6 cells) were induced for 1 h with lipopolysaccharide (100 ng/ml) and IFN- γ (500 U/ml), and total RNA was pre-

pared with TriReagent as specified by the manufacturer. Reverse transcription (RT)-PCR analysis was performed on 1 μ g of total RNA by annealing with 250 ng of a (5:1) mixture of random and oligo(dT) primers followed by incubation with reverse transcriptase (SuperScript II, Gibco-BRL) in 5 $\times$ reaction buffer for 30 min at 37°C. The reaction mixture was diluted to a final volume of 100 μ l with distilled H₂O and heat inactivated at 95 to 99°C for 5 min. PCR was carried out in a thermal cycler (Perkin-Elmer Cetus) with 2 μ l of the RT reaction mixture in a 50- μ l volume for 35 cycles, with the following forward and reverse primers: NGFI-A exon 1 fwd 1, 5'-ACTACCCAACTGGAGGAG-3'; NGFI-A exon 2 rev 2, 5'-CTACGACTGAAGTTACGCAT-3'; NGFI-A exon 2 fwd 3, 5'-ACCCGGCCAGCAAGACACC-3'; NGFI-A exon 2 rev 4, 5'-GGGCACAGGGGATGGGAATG-3'; β -actin fwd, 5'-TGAAGTACCAACATCACC-3'; β -actin rev, 5'-ACTATCACACTTGCCACAGT-3'. The reaction mixtures were electrophoresed in a 2% agarose gel, blotted with Zeta Probe membrane (Bio-Rad), probed with a 32 P-random-labeled fragment consisting of the zinc finger DNA-binding domain (NGFI-A primer set 3/4) or actin, and exposed to X-ray film (Kodak).

In vitro differentiation of marrow cultures. At 3 days after injection with 2 ml of 10% sodium caseinate to enrich for myeloblasts (17), the mice were sacrificed by cervical dislocation for collection of bone marrow cells from the femurs and tibia. Bone marrow cells (5×10^5 cells per ml) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, penicillin and streptomycin, 50 μ M 2-mercaptoethanol, and 10 ng of either G-CSF, GM-CSF, or M-CSF (R&D Research) per ml at 37°C under 5% CO₂. After 4 days, adherent cells were harvested by incubation in 0.025% trypsin-1.5 mM EDTA and pooled with nonadherent cells. The cells were washed and resuspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 0.1% NaN₃ and transferred onto glass slides by cytopsin (Shandon). Differential counts were performed for macrophages and granulocytes as identified by May-Grunwald-Giemsa stain or by α -naphthyl or specific esterase staining (Sigma) performed as specified by the manufacturer.

Flow cytometric analysis. Cells (1×10^5 to 5×10^5) were blocked by a 15-min incubation at 4°C in 200 μ l of blocking medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10% mouse serum, and 0.1% NaN₃). The cells were then stained for 30 min at 4°C with 1 μ g of either rat anti-F4/80 (CalTag), biotinylated RB6.8C5 (CalTag), or biotinylated anti-I-A^b (Pharmingen) and washed three times in phosphate-buffered saline (PBS). A secondary stain was performed with 1 μ g of either fluorescein-isothiocyanate-conjugated goat anti-rat antibody (Jackson Immunochemicals) or phycoerythrin-conjugated streptavidin (CalTag). Flow-cytometric analysis was performed with a FACScan (Becton Dickinson) and Consort 30 software.

Nitric oxide assays. Resident or thioglycolate-elicited peritoneal macrophages were isolated by peritoneal lavage with RPMI 1640 medium (10 ml). The cells (1×10^5 to 5×10^5) were plated in 96-well culture plates. The macrophages were allowed to adhere to the tissue culture surface for 4 h, and nonadherent cells were removed with three washes with medium. The macrophages were incubated for 48 h in the presence or absence of lipopolysaccharide (100 ng of *Escherichia coli* serotype O127:B8 [Sigma] per ml) and IFN- γ (a gift from R. Schrieber, Washington University), after which medium was collected and stored at -20°C until analysis. The NO₂⁻ concentration was determined by mixing medium with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-naphthylethylene diamine dihydrochloride) (4). After a 10-min incubation, the A₅₇₀ was measured and standardized to known concentrations of sodium nitrite.

***L. monocytogenes* challenge assay.** Mice were given an intraperitoneal inoculum of *L. monocytogenes* (23) and sacrificed 4 days later by cervical dislocation. Their spleens were homogenized in PBS containing 0.05% Triton X-100. Serial dilutions of the homogenate were plated onto brain heart infusion agar (Difco, Detroit, Mich.). Bacterial colonies were counted after 24 h of growth at 37°C.

Immunohistochemistry for F4/80. Mice were sacrificed by anesthetic overdose and perfused with buffer (10 mM phosphate buffer [pH 7.4], 0.85% NaCl, 0.025% KCl, 0.05% NaHCO₃) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.5). The tissues were postfixed in 4% paraformaldehyde for 2 h and cryoprotected in 30% sucrose in PBS for 12 h before being embedded in OCT (TissueTek). Cryostat sections (12 μ m) were mounted on glass slides, incubated with F4/80 cell culture supernatant (a gift from H. Virgin, Washington University) at 25°C overnight, and then subjected to a secondary incubation with a biotinylated secondary antibody and avidin-biotinylated horseradish peroxidase complex (Vectastain ABC elite kit; Vector Laboratories). Immunolabeled cells were visualized by development with diaminobenzidine-hydrogen peroxide substrate essentially as described previously (8).

RESULTS AND DISCUSSION

To investigate the requirement for NGFI-A in the monocyte/macrophage lineage, we examined mice in which NGFI-A was disrupted by gene targeting (12). Insertion of a neomycin resistance cassette within the NGFI-A gene introduces several stop codons within the first zinc finger of the DNA-binding domain. Although the mutation was verified in double-mutant ES cells by protein and RNA blotting (12), we confirmed the

FIG. 2. Evidence that macrophages are present in a variety of tissues and immune compartments. (A) Flow-cytometric analysis of cells from the peritoneal cavity and spleen labeled with the macrophage marker F4/80. (B to I) Immunohistochemical labeling with the macrophage marker F4/80 of tissue sections of the liver (B and C), spleen (D and E), uterus (F and G), and testis (H and I) from wild-type (B, D, F, and H) and NGFI-A^{-/-} (C, E, G, and I) mice. In the spleen, positive staining was detected primarily in the red pulp (r) and not the white pulp (w). Bar, 100 μ m.

mutation in peritoneal exudate cells derived from these ES cells. RT-PCR was used to detect potential aberrant splice products resulting from excision of the *neo* cassette and fusion of either exon 1 or the 5' part of exon 2 with the remainder of exon 2 containing part of the DNA-binding domain. As shown in Fig. 1, no aberrant splice products were detected in PCRs of homozygous mutant peritoneal exudate cells. These results confirm our previous analyses indicating that the resultant mutation is a null mutation, and we therefore refer to the homozygous NGFI-A mutation as NGFI-A^{-/-}.

The effect of NGFI-A deficiency on the monocyte/macrophage lineage was initially investigated by examination of May-Grunwald-Giemsa-stained smears of peripheral blood from NGFI-A^{-/-} mice. This revealed monocytes with normal morphology and with a similar differential proportion to that in wild-type mice (between 1 and 3%). Quantitation of macrophages in the peritoneal cavity and spleen by flow cytometry with the cell surface marker F4/80 (7, 8) revealed that the relative proportion of macrophages in these compartments was also similar in wild-type and NGFI-A^{-/-} mice (Fig. 2A). Examination by immunohistochemical analysis of other macrophage compartments including liver, spleen, uterus, and testis revealed a distribution of macrophages that was similar between wild-type, NGFI-A^{+/+} and NGFI-A^{-/-} mice (Fig. 2B to I).

We next examined the effect of NGFI-A deficiency on cultured bone marrow cells induced to undergo myeloid cell differentiation. After culture of bone marrow cells of either wild-type or NGFI-A^{-/-} mice in the presence of either M-CSF, GM-CSF, or G-CSF, macrophage and granulocyte differenti-

ation was assessed by differential counts of May-Grunwald-Giemsa-stained cytopsin preparations. In cultures containing M-CSF, both wild-type and NGFI-A^{-/-} bone marrow cells produced macrophages with their characteristic vacuolated cytoplasm and lightly stained chromatin. In cultures containing GM-CSF, both wild-type and NGFI-A^{-/-} bone marrow cells yielded a mixed population of macrophages and granulocytes, with similar differential proportions of each (Fig. 3A to D). Macrophages derived from both M-CSF and GM-CSF also stained positive for the cytochemical marker α -naphthyl esterase (data not shown). In contrast, G-CSF cultures of either wild-type or NGFI-A^{-/-} bone marrow cells yielded predominantly granulocytes (Fig. 3E and F).

While these results indicate that NGFI-A is not required for macrophage differentiation, we considered the possibility that a compensatory increase in the number of progenitor stem cells in the bone marrow masked a slight qualitative difference in macrophage differentiation. We therefore used methylcellulose culture assays to determine the number of progenitor stem cells. Culture of bone marrow cells for 5 days in the presence of M-CSF and G-CSF resulted in a similar number of colonies for cells from wild-type and NGFI-A^{-/-} mice (Fig. 4). The colonies that formed in the presence of M-CSF contained comparable numbers of cells that, when plated into liquid medium, produced adherent cells with typical macrophage morphology.

Although we did not observe a defect in macrophage differentiation, the association of NGFI-A induction with macrophage activation suggested the possibility that NGFI-A is required for specific functions of activated macrophages. We

FIG. 2—Continued.

FIG. 3. M-CSF-, GM-CSF-, and G-CSF-induced differentiation proceeds normally in bone marrow cells derived from NGFI-A^{-/-} mice. Representative cytospin smears of wild-type (A, C, and E) and NGFI-A^{-/-} (B, D, and F) bone marrow cells cultured for 5 days in the presence of M-CSF (10 ng/ml) (A and B), GM-CSF (10 ng/ml) (C and D), or G-CSF (10 ng/ml) (E and F) (May-Grunwald-Giemsa stain; bar, 75 μ m). The relative proportion of identifiable macrophages and granulocytes determined by differential counting was found to be similar in wild-type and NGFI-A^{-/-} samples. The experiments were repeated twice with similar results.

therefore examined various parameters of macrophage activation including the ability to produce nitric oxide in response to lipopolysaccharide and IFN- γ , the ability to express class II MHC molecules, and the clearance of pathogens from infected mice. To examine NO production, thioglycolate-elicited peritoneal exudate cells of wild-type and NGFI-A^{-/-} mice were cultured in the presence or absence of lipopolysaccharide and IFN- γ for 48 h. As shown in Fig. 5A, the production of NO was similar for both wild-type and NGFI-A^{-/-} macrophages.

As another measure of macrophage activation, we assessed the expression of class II MHC molecules, which is normally low but is induced upon infection by *L. monocytogenes*. Flow-cytometric analysis of class II MHC molecule expression revealed that peritoneal macrophages from uninfected mice

were characteristically I-A^{low} and F4/80^{hi}. After infection with *L. monocytogenes*, the macrophage subpopulation was predominantly F4/80^{lo} and I-A^{hi}. This increase in expression was similar in both wild-type and NGFI-A^{-/-} mice (Fig. 5B).

It has been shown previously that the ability of activated macrophages to stimulate T cells is required for clearance of listeriae and for ultimate sterilizing immunity. Intraperitoneal infection of NGFI-A^{-/-} mice with a sublethal dose of *L. monocytogenes* (10⁴ CFU) demonstrated no increased susceptibility to infection, because these mice survived for several weeks. The ability of NGFI-A^{-/-} mice to clear the organism was assessed by determining the bacterial burden in the spleen 4 days after infection (Fig. 6). NGFI-A^{-/-} mice had bacterial levels comparable to those present in wild-type mice; more-

FIG. 4. Functional analysis of bone marrow cells from wild-type and NGFI-A^{-/-} mice. Bone marrow cells in methylcellulose suspension were cultured for 5 days in the presence of either M-CSF or G-CSF, and the number of colonies was counted in duplicate wells. Data represent the mean and standard deviation of three independent experiments.

FIG. 6. Bacterial burden in the spleens of wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mice 4 days after challenge with *L. monocytogenes* infection (10⁴ CFU). The data represent the mean and standard deviation for three mice of each genotype.

over, the bacterial burden disappeared completely from mice infected for a prolonged period (7 days) (data not shown), indicating that sterilizing immunity provided by the T-cell response was intact in NGFI-A^{-/-} mice.

In contrast to previous loss-of-function studies, which used antisense oligonucleotide approaches, we have found no requirement for NGFI-A in macrophage differentiation. We considered several possible explanations for this discrepancy. Although functional redundancy by coexpression of related

proteins Krox-20 or other members of the NGFI-A family is often put forth to explain these disparate results, this cannot account for the observed effects in the antisense oligonucleotide experiments, because they presumably result from reduced expression of the specific target gene. One possibility is that early in development, the plasticity of signal transduction mechanisms enables bone marrow cells from NGFI-A^{-/-} mice to adapt to NGFI-A deficiency through alternate NGFI-A-independent mechanisms such that these cells no longer require NGFI-A.

FIG. 5. Assessment of macrophage activation by nitric oxide production (A) and class II MHC expression (B). (A) IFN- γ -induced nitric oxide production from wild-type and NGFI-A^{-/-} thioglycolate-elicited peritoneal macrophages. The data represent the mean and standard deviation for four mice of each genotype. (B) Cytofluorometric analysis of peritoneal exudate cells derived from untreated mice (-LM) and from mice infected 3 days earlier with a 10⁴ CFU intraperitoneal injection of *L. monocytogenes* (+LM). The two-dimensional plots indicate I-A class II MHC expression (red fluorescence) in cells which were gated on the basis of side scatter and positive F4/80 staining (green fluorescence). The results shown are representative of two independent experiments.

Alternatively, the discrepancy between gene knockout and antisense oligonucleotide experiments could be explained by "non-antisense" effects, a phenomenon frequently reported in antisense oligonucleotide experiments (1). Interestingly, most of the experimentally effective oligonucleotides directed against NGFI-A contain four contiguous guanosine residues, a characteristic which has been demonstrated to have antiproliferative and antidifferentiative properties. In addition to the decreased expression of NGFI-A, such non-antisense effects may contribute to the observed block in macrophage differentiation.

We also considered the possibility that the knockout mutation was leaky because of possible alternative splicing, stop codon readthrough, or other mechanisms. RT-PCR analysis failed to demonstrate the existence of alternatively spliced products. We have shown previously by enhanced chemiluminescent immunoblot analysis that ES cells homozygous for the mutation did not produce any detectable NGFI-A (12). These results were confirmed with cells derived from NGFI-A^{-/-} mice as well.

Finally, it remains possible that a subtle defect in macrophage function exists in NGFI-A^{-/-} mice. Several genes originally identified as inducible during differentiation of myeloid leukemia cell lines were later shown in knockout experiments to be dispensable for myeloid cell differentiation but were required for a more distal function in the macrophage lineage. A striking example is that of *c-fos*, which was identified in early experiments as an induced gene in HL-60 and many other cell lines. Targeted mutagenesis of *c-fos* demonstrated that it was nonessential for many of the processes associated with its expression such as B- and T-cell activation, proliferation, or apoptosis (9, 10, 18, 26) but proved essential for differentiation of osteoclasts, cells derived from the macrophage lineage (6). However, to date, we have been unable to demonstrate a loss of any particular subpopulation of macrophages in NGFI-A^{-/-} mice and therefore conclude that NGFI-A is not required for macrophage differentiation and activation.

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