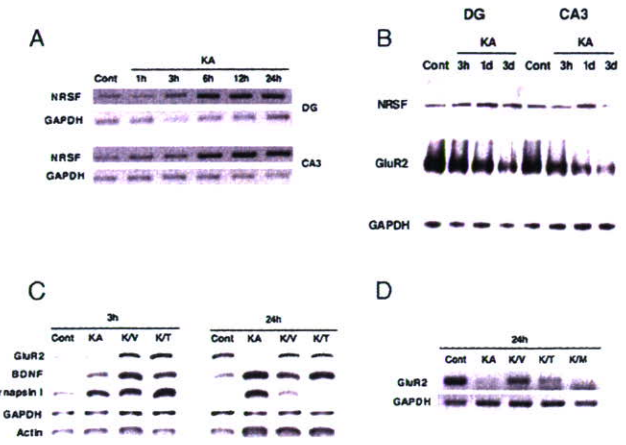


Figure 4. VPA inhibits seizure-mediated neurogenesis by normalizing changes in gene expression. ***A–D***, RT-PCR and Western blot analysis of DG and CA3 from saline-injected (Cont) and KA-injected (KA) rats from 1 h to 3 d (***A***, ***B***). RT-PCR of DG and CA3 from control (Cont) and seizure (KA) rats from 1–24 h (***A***). Representative Western blots of DG and CA3 from control (Cont) and seizure (KA) animals from 3 h to 3 d (***B***). RT-PCR of DG from saline-injected (Cont) and KA-injected animals receiving either VPA (K/V) or TSA (K/T) (1 h before treatment) and RNA harvested 3 and 24 h after onset of seizures (***C***). RT-PCR of DG from control (Cont) and KA-treated rats with VPA (K/V), TSA (K/T), or valpromide (K/M) given 5 h after seizure onset, and RNA was harvested 24 h later (***D***). GAPDH and/or β-actin (data not shown) were used as normalization controls. Data are representative of at least three independent experiments.

Neuronal cell death does not appear to be affected by VPA

These results indicated that VPA potently inhibited seizure-induced, aberrant neurogenesis by normalizing HDAC-regulated gene expression. Recent studies have suggested that VPA has a neuroprotective effect after traumatic brain injury (Morland et al., 2004; Ren et al., 2004). Because KA-induced seizures result in a considerable amount of neuronal cell death (Ben-Ari and Cossart, 2000; Bengzon et al., 2002), we next analyzed whether VPA had a neuroprotective effect after KA-induced seizures, in addition to its inhibition of seizure-induced neurogenesis. We stained sections from all rats of the survival groups with Fluoro-Jade B, which labels degenerating neurons (Schmued and Hopkins, 2000). Careful visual analysis by two blinded investigators did not reveal any significant differences in the amount of neuronal cell death, observed throughout the brains of seizure animals that did or did not receive VPA injections (Fig. 5*A–D*). Nevertheless, we sought unbiased, quantitative numbers of surviving neurons. Therefore, we stereologically assessed cell densities of the hippocampus in all rats that were killed 5 weeks after seizure induction. Confirming previous results, we found decreased neuronal cell densities within the DG in all rats that had experienced SE (Fig. 5*E*) (Holtkamp et al., 2005). Importantly, the VPA-treated animals showed a comparable degree of neuronal degeneration in the dentate area as the untreated KA-injected animals (Fig. 5*E*,*F*). In addition, we did not detect any neuroprotective effect of VPA on pyramidal cells in the cornu ammonis (data not shown). In summary, VPA treatment did not result in a substantial neuroprotective effect after KA-induced seizures in the adult rat.

Improved hippocampal function in VPA-treated, KA-injected animals

Prolonged seizure activity eventually leads to severe impairment in cognition and in learning and memory in humans but also in rodent models of temporal lobe epilepsy (TLE) (Stafstrom et al., 1993; Holmes, 1997; Helmstaedter, 2002; Stefan and Pauli, 2002; Elger et al., 2004). VPA treatment blocked aberrant neurogenesis after KA-induced SE and thus inhibited the excess addition of seizure-generated granule cells into the preexisting dentate circuitry. Given this finding, we hypothesized that VPA treatment may protect KA-treated animals from cognitive impairment by inhibiting the addition of new, aberrant neurons to the adult GCL. We tested all rats in the survival groups 5 weeks after seizure induction in a hippocampus-dependent object recognition test (Clark et al., 2000). All groups showed comparable levels of exploratory behavior (Fig. 6*A*), and we found no preference for one of the objects used in this experiment (data not shown). After a 3 h delay, a new object was introduced into the testing chamber together with an identical copy of the familiar object. Control animals showed a strong preference to explore the new object within the first 30 s of exploration (Fig. 6*B*). In clear contrast, rats that had experienced KA-induced seizures spent the same amount of time exploring the familiar object as they did exploring the new object, indicating impairment in hippocampus-dependent object memory (Fig. 6*B*). Strikingly, this phenotype was completely prevented in the VPA-treated KA-injected animals, which showed a preference toward the novel object similar to controls (Fig. 6*B*) ($p < 0.001$; $F = 27.3$). Given this finding, VPA treatment protected KA-injected animals from the cognitive impairment that was detectable in untreated KA-injected animals in a hippocampus-dependent object recognition test. Because previous studies had shown that the increase in proliferative activity occurred mainly during the first 14 d after SE (Parent et al., 1997) and to strengthen the association between seizure-induced neurogenesis and subsequent memory impairment, we restricted, in a separate experiment, the duration of VPA injections to the first 2 weeks after KA injection. Paralleling our first experiment, we found a robust inhibition of seizure-induced neurogenesis by short-term VPA treatment (in this experiment, the animals were killed 2 and 4 weeks after SE). Strikingly, treatment for 14 d after SE with VPA also prevented seizure-associated memory impairment (tested 4 weeks after SE) at a comparable level with the long-term VPA treatment (data not shown).

Discussion

Here we show that aberrant neurogenesis induced by seizure activity within the hippocampal circuitry was potently blocked by the antiepileptic drug VPA. The efficacy of VPA in decreasing the neurogenic response to seizure activity appeared to be mediated by VPA-induced inhibition of HDACs and subsequent normalization of HDAC-dependent gene expression. Strikingly, animals that had experienced SE, but received VPA treatment, did not show seizure-associated cognitive impairment in a hippocampus-dependent object recognition test.

After decades of controversy, the finding that new neurons are added into the hippocampal circuitry throughout life is now broadly accepted (Gage, 2002). However, the functional significance of adult neurogenesis remains unclear (Kempermann, 2002; Schinder and Gage, 2004). Supporting a functional role for physiologically generated new granule cells is the finding that ablation of new neurons with cytostatic drugs or irradiation correlates with impairment in some hippocampus-dependent learning tasks (Shors et al., 2001, 2002; Snyder et al., 2005), although the specificity of the above-mentioned ablation techniques (Monje et al., 2002) is subject to controversy within the field of adult neurogenesis (Dupret et al., 2005). Interestingly, seizure activity within the hippocampal formation induces the formation of new granule cells, apparently without having a beneficial effect on learning and memory. Given this finding, it has been specu-

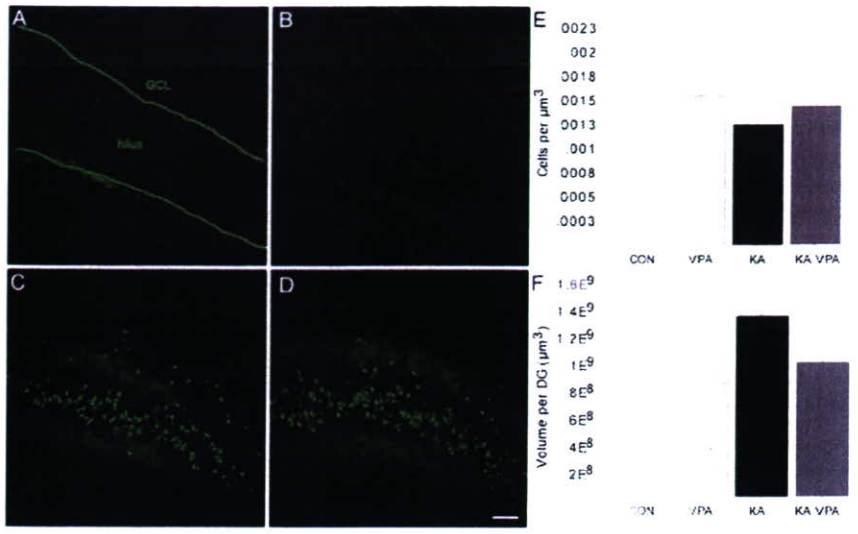


Figure 5. Seizure-induced cell death in the dentate area is not affected by VPA treatment. ***A–D***, Fluoro-Jade B staining of saline (***A***), VPA-treated (***B***), KA-injected (***C***), and KA-injected plus VPA-treated (***D***) animals killed 5 weeks after SE shows degenerating neuronal cells in the hilus in the KA-injected animals. ***E***, ***F***, Cell density and DG volume in control animals and seizure animals 5 weeks after SE. Scale bar, 100 μm.

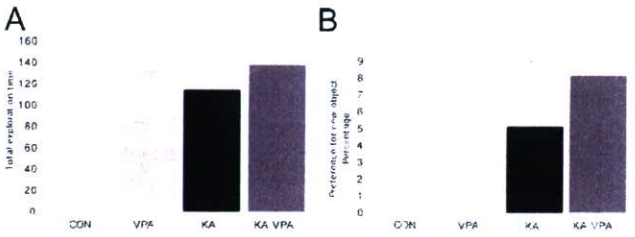


Figure 6. Seizure-associated cognitive impairment is prevented by VPA treatment. ***A***, ***B***, Although the total exploration time was comparable between all groups (***A***), KA-challenged animals did not show a preference for the new object 3 h after the presentation of the initial objects (***B***). VPA treatment prevented this phenotype, and KA-challenged animals that received VPA injections showed the same level of preference for the new objects as controls did. $*p < 0.01$.

lated that new neurons generated in response to epileptic activity may contribute to the pathological consequences of SE rather than represent an attempt by the injured brain to replace lost cells (Parent, 2002; Parent and Lowenstein, 2002). What distinguishes seizure-induced neurogenesis from physiological neurogenesis? Furthermore, might certain antiepileptic drugs modulate seizure-induced neurogenesis?

Seizure activity leads to increased proliferative activity of DCX-positive dividing cells, presumably representing committed neuroblasts (Jessberger et al., 2005). Currently, the consequences of increased activity of DCX-positive cells remain to be characterized (Jessberger et al., 2005). Nevertheless, this abnormal activation of late progenitors was completely absent in rats treated with the antiepileptic drug VPA. Furthermore, the hilar extension of basal dendrites arising from seizure-generated granule cells was prevented by VPA treatment of KA-injected animals. Previous studies suggested that extensive basal dendrites might contribute to recurrent excitatory circuits interfering with normal synaptic transmission (Ribak et al., 2000; Dashtipour et al., 2003; Overstreet-Wadiche et al., 2006; Shapiro and Ribak, 2006).

In striking contrast to the KA and pilocarpine rat and mouse model of TLE that are all associated with dramatic morphologic changes of seizure-induced granule cells (Parent et al., 1997; Scharfman et al., 2000; Overstreet-Wadiche et al., 2006; Shapiro et al., 2006), Jakubs et al. (2006) recently reported that granule cells that are born after SE induced by electrical stimulation are less excitable than granule cells born in running rats. Jakubs and colleagues speculated that decreased excitability of seizure-induced cells might be an attempt of the injured brain to restore brain homeostasis. The reasons for the discrepancy between their findings (regular morphology) and other reports (extensive changes in granule cell morphology after SE) remain unknown but might be explained by the different seizure models used in the respective studies.

Besides the normalization of seizure-associated morphological changes after KA-induced seizures, we found a significant reduction in the number of seizure-generated neurons in VPA-treated animals. This effect of VPA appeared to be mainly mediated by inhibition of seizure-induced cell proliferation. Indeed, the cyclin-dependent inhibitor p21WAF1/CIP1 is a common target induced by short-chain fatty acid HDAC inhibitors such as VPA and sodium butyrate (Gurvich et al., 2004) and is rapidly induced with VPA treatment after KA treatment (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Previously, our group reported that VPA and other HDAC inhibitors have the ability to block adult hippocampal neural progenitor cell proliferation and promote neuronal and suppress glial differentiation, respectively (Hsieh et al., 2004). The mechanism of VPA as an antiepileptic has remained elusive (Owens and Nemeroff, 2003). Two possible models to explain the nature of its effects on blocking aberrant neurogenesis include a direct versus indirect mode of action. An example of an indirect course of action is that VPA blocks seizure activity, possibly through the modulation of GABA levels, and the reduction of seizure activity and/or recurrent seizures leads to the decrease in aberrant neurogenesis. We think that several arguments speak against the explanation that VPA injections simply reduce initial seizure activity. First of all, we injected the first VPA dose 5 h after the onset of SE. At that time, behavioral seizure activity had already been terminated. Previous studies also showed that even milder seizures induce the maximum amount of neurogenic cell proliferation (Mohapel et al., 2004). Second, our EEG data suggested that VPA does not significantly alter seizure strength or frequency in seizure rats compared with saline controls early after KA injection.

Thus, we favor the alternative model: the actions of VPA directly inhibit HDACs and normalize changes in gene expression after seizures. Among the numerous genes that change after seizure induction (Elliott et al., 2003; Majores et al., 2004), it was reported recently that, after pilocarpine-induced SE, changes in two NRSF-regulated neuronal genes, GluR2 and BDNF, could be prevented by the HDAC inhibitor TSA (Huang et al., 2002). Because there is also a rapid induction of NRSF mRNAs in the hippocampus after seizures (Palm et al., 1998), NRSF may function as a context-dependent regulator of gene expression in both neurons and non-neuronal cells. Immunohistochemistry experiments revealed detectable levels of NRSF in the dentate granule cell layer of adult rat hippocampus (supplemental Fig. 2, available

at www.jneurosci.org as supplemental material). The specific cell types that differentially express NRSF after seizure activity remain unknown. Nevertheless, modulation of NRSF expression and/or activity [i.e., by small noncoding RNAs matching the same sequence as the NRSF binding site called *NRSE* smRNAs (Kuwabara et al., 2004)] could be a critical event in seizure-mediated neurogenesis. Additional experiments are needed to determine whether NRSF gene expression and/or NRSF protein activity become dysregulated after SE and whether VPA and other HDAC inhibitors act on the expression of NRSF itself, which in turn might affect downstream target genes or act directly on the expression of *NRSE*-containing neuronal genes. In fact, a recent study has found that VPA treatment could significantly reduce seizure-induced expression of two immediate early genes, c-fos and c-Jun, reinforcing the idea that VPA belongs to a class of antiepileptic drugs that normalizes the expression of seizure-induced genes (Szot et al., 2005). What might be the result of aberrant seizure-induced gene expression? Simplified, two features reflecting human pathology are associated with the KA rodent model of human TLE: epileptogenesis (the occurrence of spontaneous seizures after a latent period) and cognitive impairment (Helmstaedter et al., 2003). Altered gene expression within mature hippocampal neurons but also aberrant gene expression that results in disturbed neurogenesis may be responsible for these two consequences of prolonged epileptic activity within the hippocampal circuitry. Here we report that VPA treatment robustly prevented seizure-induced neurogenesis and protected animals that had experienced SE from seizure-associated impairment in a hippocampus-dependent learning task. To assess hippocampal function, we used an object recognition task with a time interval between the presentation of a new object relative to the familiarization that depends on intact hippocampal function in rodents (Clark et al., 2000). Rats that had experienced KA-induced SE showed a severe impairment in this test. This finding was in striking contrast to the performance of SE animals that had received VPA injections. Interestingly, it has been reported previously that patients with TLE display deficits in recognition memory (for review, see Elger et al., 2004). In addition, a previous study showed the prevention of cognitive impairment after KA-induced seizures by VPA treatment using the Morris water maze (Bolanos et al., 1998). Therefore, the inhibition of aberrant seizure-induced neurogenesis and the morphological normalization of neurons that were generated in response to SE by VPA treatment might at least partially explain the observed protection from seizure-associated cognitive impairment. At this point, we cannot determine the relative impact of the increased numbers or altered morphology of seizure-generated neurons on cognitive performance after SE.

Given the variety of cellular and molecular changes after SE, it is clear that KA-induced aberrant neurogenesis is not the sole cause of cognitive impairment associated with seizure activity. Therefore, ongoing studies will address the functional consequences of seizure-generated neurons on a single-cell level and the effects of aberrant neurons on hippocampal synaptic transmission. In addition, future ablation studies will further characterize the relative contribution of seizure-induced neurogenesis to impaired learning and memory after SE.

References

Ballas N, Battaglioli E, Atouf F, Andres ME, Chenoweth J, Anderson ME, Burger C, Moniwa M, Davie JR, Bowers WJ, Federoff HJ, Rose DW, Rosenfeld MG, Brehm P, Mandel G (2001) Regulation of neuronal traits by a novel transcriptional complex. Neuron 31:353–365.

Ben-Ari Y, Cossart R (2000) Kainate, a double agent that generates seizures: two decades of progress. Trends Neurosci 23:580–587.

Bengzon J, Kokaia Z, Elmér E, Nanobashvili A, Kokaia M, Lindvall O (1997) Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures. Proc Natl Acad Sci USA 94:10432–10437.

Bengzon J, Mohapel P, Ekdahl CT, Lindvall O (2002) Neuronal apoptosis after brief and prolonged seizures. Prog Brain Res 135:111–119.

Beuckmann CT, Sinton CM, Williams SC, Richardson JA, Hammer RE, Sakurai T, Yanagisawa M (2004) Expression of a poly-glutamine-ataxin-3 transgene in orexin neurons induces narcolepsy-cataplexy in the rat. J Neurosci 24:4469–4477.

Bolanos AR, Sarkisian M, Yang Y, Hori A, Helmers SL, Mikati M, Tandon P, Stafstrom CE, Holmes GL (1998) Comparison of valproate and phenobarbital treatment after status epilepticus in rats. Neurology 51:41–48.

Brown JP, Couillard-Despres S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG (2003) Transient expression of doublecortin during adult neurogenesis. J Comp Neurol 467:1–10.

Buckmaster PS, Dudek FE (1999) In vivo intracellular analysis of granule cell axon reorganization in epileptic rats. J Neurophysiol 81:712–721.

Chong JA, Tapia-Ramirez J, Kim S, Toledo-Aral JJ, Zheng Y, Boutros MC, Altshuller YM, Frohman MA, Kraner SD, Mandel G (1995) REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. Cell 80:949–957.

Clark RE, Zola SM, Squire LR (2000) Impaired recognition memory in rats after damage to the hippocampus. J Neurosci 20:8853–8860.

Couillard-Despres S, Winner B, Schaubeck S, Aigner R, Vroemen M, Weidner N, Bogdahn U, Winkler J, Kuhn HG, Aigner L (2005) Doublecortin expression levels in adult brain reflect neurogenesis. Eur J Neurosci 21:1–14.

Dashtipour K, Wong AM, Obenaus A, Spigelman I, Ribak CE (2003) Temporal profile of hilar basal dendrite formation on dentate granule cells after status epilepticus. Epilepsy Res 54:141–151.

Dupret D, Montaron MF, Drapeau E, Aurousseau C, Le Moal M, Piazza PV, Abrous DN (2005) Methylazoxymethanol acetate does not fully block cell genesis in the young and aged dentate gyrus. Eur J Neurosci 22:778–783.

Elger CE, Helmstaedter C, Kurthen M (2004) Chronic epilepsy and cognition. Lancet Neurol 3:663–672.

Elliott RC, Miles MF, Lowenstein DH (2003) Overlapping microarray profiles of dentate gyrus gene expression during development- and epilepsy-associated neurogenesis and axon outgrowth. J Neurosci 23:2218–2227.

Friedman LK, Pellegrini-Giampietro DE, Sperber EF, Bennett MV, Moshe SL, Zukin RS (1994) Kainate-induced status epilepticus alters glutamate and $GABA_A$ receptor gene expression in adult rat hippocampus: an *in situ* hybridization study. J Neurosci 14:2697–2707.

Gage F (2000) Mammalian neural stem cells. Science 287:1433–1438.

Gage FH (2002) Neurogenesis in the adult brain. J Neurosci 22:612–613.

Gastaldi M, Bartolomei F, Massacrier A, Planells R, Robaglia-Schlupp A, Cau P (1997) Increase in mRNAs encoding neonatal II and III sodium channel alpha-isoforms during kainate-induced seizures in adult rat hippocampus. Brain Res Mol Brain Res 44:179–190.

Gerdes J, Schwab U, Lemke H, Stein H (1983) Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 31:13–20.

Golden GT, Smith GG, Ferraro TN, Reyes PF (1995) Rat strain and age differences in kainic acid induced seizures. Epilepsy Res 20:151–159.

Gottlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG, Heinzel T (2001) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 20:6969–6978.

Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ (1999) Learning enhances adult neurogenesis in the hippoampal formation. Nat Neurosci 2:260–265.

Grooms SY, Opitz T, Bennett MV, Zukin RS (2000) Status epilepticus decreases glutamate receptor 2 mRNA and protein expression in hippocampal pyramidal cells before neuronal death. Proc Natl Acad Sci USA 97:3631–3636.

Gurvich N, Tsygankova OM, Meinkoth JL, Klein PS (2004) Histone deacetylase is a target of valproic acid-mediated cellular differentiation. Cancer Res 64:1079–1086.

Helmstaedter C (2002) Effects of chronic epilepsy on declarative memory systems. Prog Brain Res 135:439–453.

Helmstaedter C, Kurthen M, Lux S, Reuber M, Elger CE (2003) Chronic epilepsy and cognition: a longitudinal study in temporal lobe epilepsy. Ann Neurol 54:425–432.

Henry TR (2003) The history of valproate in clinical neuroscience. Psychopharmacol Bull 37 [Suppl 2]:5–16.

Holmes GL (1997) Epilepsy in the developing brain: lessons from the laboratory and clinic. Epilepsia 38:12–30.

Holtkamp M, Matzen J, van Landeghem F, Buchheim K, Meierkord H (2005) Transient loss of inhibition precedes spontaneous seizures after experimental status epilepticus. Neurobiol Dis 19:162–170.

Hsieh J, Nakashima K, Kuwabara T, Mejia E, Gage FH (2004) Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. Proc Natl Acad Sci USA 101:16659–16664.

Huang Y, Doherty JJ, Dingledine R (2002) Altered histone acetylation at glutamate receptor 2 and brain-derived neurotrophic factor genes is an early event triggered by status epilepticus. J Neurosci 22:8422–8428.

Jakubs K, Nanobashvili A, Bonde S, Ekdahl CT, Kokaia Z, Kokaia M, Lindvall O (2006) Environment matters: synaptic properties of neurons born in the epileptic adult brain develop to reduce excitability. Neuron 52:1047–1059.

Jessberger S, Kempermann G (2003) Adult-born hippocampal neurons mature into activity-dependent responsiveness. Eur J Neurosci 18:2707–2712.

Jessberger S, Romer B, Babu H, Kempermann G (2005) Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells. Exp Neurol 196:342–351.

Kee N, Sivalingam S, Boonstra R, Wojtowicz JM (2002) The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J Neurosci Methods 115:97–105.

Kempermann G (2002) Why new neurons? Possible functions for adult hippocampal neurogenesis. J Neurosci 22:635–638.

Kempermann G, Gage FH (1999) Experience-dependent regulation of adult hippocampal neurogenesis: effects of long-term stimulation and stimulus withdrawal. Hippocampus 9:321–332.

Kempermann G, Kuhn HG, Gage FH (1997) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493–495.

Kuwabara T, Hsieh J, Nakashima K, Taira K, Gage FH (2004) A small modulatory dsRNA specifies the fate of adult neural stem cells. Cell 116:779–793.

Majores M, Eils J, Wiestler OD, Becker AJ (2004) Molecular profiling of temporal lobe epilepsy: comparison of data from human tissue samples and animal models. Epilepsy Res 60:173–178.

Mohapel P, Ekdahl CT, Lindvall O (2004) Status epilepticus severity influences the long-term outcome of neurogenesis in the adult dentate gyrus. Neurobiol Dis 15:196–205.

Monje ML, Mizumatsu S, Fike JR, Palmer TD (2002) Irradiation induces neural precursor-cell dysfunction. Nat Med 8:955–962.

Morland C, Boldingh KA, Iversen EG, Hassel B (2004) Valproate is neuroprotective against malonate toxicity in rat striatum: an association with augmentation of high-affinity glutamate uptake. J Cereb Blood Flow Metab 24:1226–1234.

Naruse Y, Aoki T, Kojima T, Mori N (1999) Neural restrictive silencer factor recruits mSin3 and histone deacetylase complex to repress neuron-specific target genes. Proc Natl Acad Sci USA 96:13691–13696.

Overstreet-Wadiche LS, Bromberg DA, Bensen AL, Westbrook GL (2006) Seizures accelerate functional integration of adult-generated granule cells. J Neurosci 26:4095–4103.

Owens MJ, Nemeroff CB (2003) Pharmacology of valproate. Psychopharmacol Bull 37 [Suppl 2]:17–24.

Palm K, Belluardo N, Metsis M, Timmusk T (1998) Neuronal expression of zinc finger transcription factor REST/NRSF/XBR gene. J Neurosci 18:1280–1296.

Parent JM (2002) The role of seizure-induced neurogenesis in epileptogenesis and brain repair. Epilepsy Res 50:179–189.

Parent JM, Lowenstein DH (2002) Seizure-induced neurogenesis: are more new neurons good for an adult brain? Prog Brain Res 135:121–131.

Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J Neurosci 17:3727–3738.

Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J Biol Chem 276:36734–36741.

Pollard H, Heron A, Moreau J, Ben-Ari Y, Khrestchatisky M (1993) Alterations of the GluR-B AMPA receptor subunit flip/flop expression in kainate-induced epilepsy and ischemia. Neuroscience 57:545–554.

Ren M, Leng Y, Jeong M, Leeds PR, Chuang DM (2004) Valproic acid reduces brain damage induced by transient focal cerebral ischemia in rats: potential roles of histone deacetylase inhibition and heat shock protein induction. J Neurochem 89:1358–1367.

Ribak CE, Tran PH, Spigelman I, Okazaki MM, Nadler JV (2000) Status epilepticus-induced hilar basal dendrites on rodent granule cells contribute to recurrent excitatory circuitry. J Comp Neurol 428:240–253.

Sanchez RM, Koh S, Rio C, Wang C, Lamperti ED, Sharma D, Corfas G, Jensen FE (2001) Decreased glutamate receptor 2 expression and enhanced epileptogenesis in immature rat hippocampus after perinatal hypoxia-induced seizures. J Neurosci 21:8154–8163.

Scharfman HE, Goodman JH, Sollas AL (2000) Granule-like neurons at the hilar/CA3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis. J Neurosci 20:6144–6158.

Schinder AF, Gage FH (2004) A hypothesis about the role of adult neurogenesis in hippocampal function. Physiology (Bethesda) 19:253–261.

Schmued LC, Hopkins KJ (2000) Fluoro-Jade: novel fluorochromes for detecting toxicant-induced neuronal degeneration. Toxicol Pathol 28:91–99.

Schoenherr CJ, Anderson DJ (1995) The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes. Science 267:1360–1363.

Scott BW, Wang S, Burnham WM, De Boni U, Wojtowicz JM (1998) Kindling-induced neurogenesis in the dentate gyrus of the rat. Neurosci Lett 248:73–76.

Shapiro LA, Ribak CE (2006) Newly born dentate granule neurons after pilocarpine-induced epilepsy have hilar basal dendrites with immature synapses. Epilepsy Res 69:53–66.

Shen S, Li J, Casaccia-Bonnefil P (2005) Histone modifications affect timing of oligodendrocyte progenitor differentiation in the developing rat brain. J Cell Biol 169:577–589.

Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E (2001) Neurogenesis in the adult is involved in the formation of trace memories. Nature 410:372–376.

Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E (2002) Neurogenesis may relate to some but not all types of hippocampal-dependent learning. Hippocampus 12:578–584.

Snyder JS, Hong NS, McDonald RJ, Wojtowicz JM (2005) A role for adult neurogenesis in spatial long-term memory. Neuroscience 130:843–852.

Stafstrom CE, Chronopoulos A, Thurber S, Thompson JL, Holmes GL (1993) Age-dependent cognitive and behavioral deficits after kainic acid seizures. Epilepsia 34:420–432.

Stefan H, Pauli E (2002) Progressive cognitive decline in epilepsy: an indication of ongoing plasticity. Prog Brain Res 135:409–417.

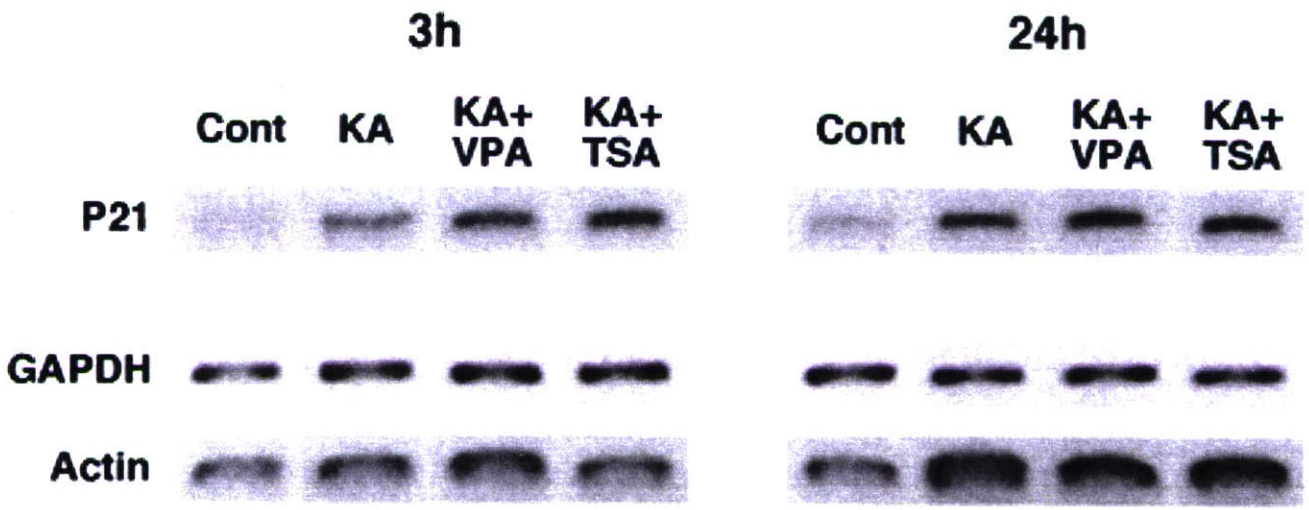
Szot P, White SS, Shen DD, Anderson GD (2005) Valproic acid, but not lamotrigine, suppresses seizure-induced c-fos and c-Jun mRNA expression. Brain Res Mol Brain Res 135:285–289.

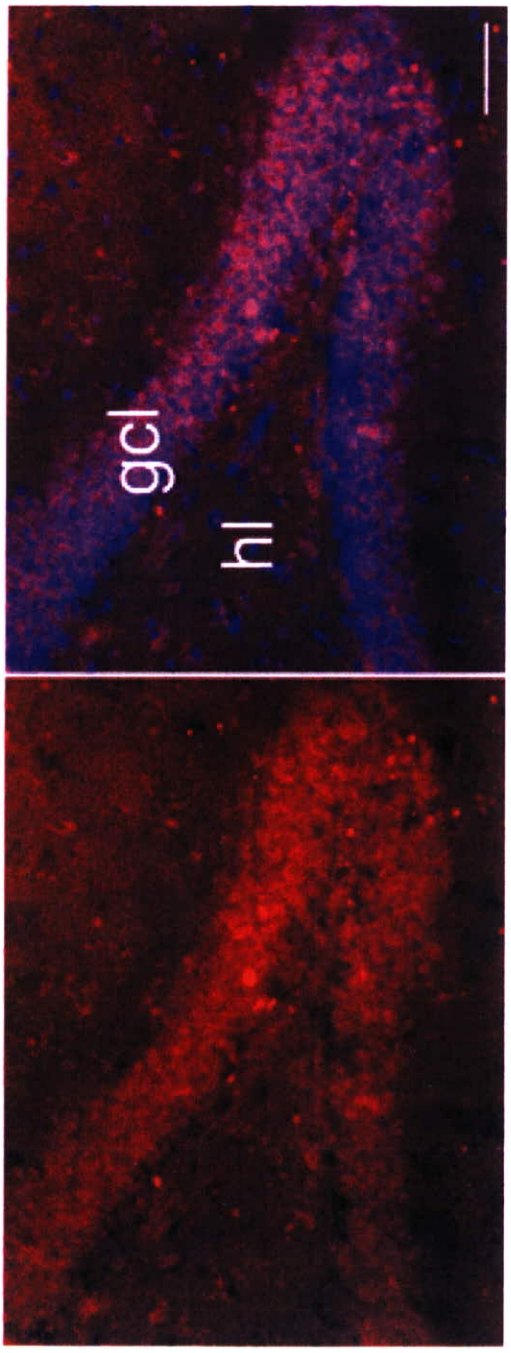
Tongiorgi E, Armellin M, Giulianini PG, Bregola G, Zucchini S, Paradiso B, Steward O, Cattaneo A, Simonato M (2004) Brain-derived neurotrophic factor mRNA and protein are targeted to discrete dendritic laminas by events that trigger epileptogenesis. J Neurosci 24:6842–6852.

Van Praag H, Kempermann G, Gage FH (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 2:266–270.

Winkler I, Blotnik S, Shimshoni J, Yagen B, Devor M, Bialer M (2005) Efficacy of antiepileptic isomers of valproic acid and valpromide in a rat model of neuropathic pain. Br J Pharmacol 146:198–208.

Zafra F, Hengerer B, Leibrock J, Thoenen H, Lindholm D (1990) Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. EMBO J 9:3545–3550.


3h
24h
Cont
KA
KA+ VPA
KA+ TSA
Cont
KA
KA+ VPA
KA+ TSA
P21
GAPDH
Actin


gcl
hl

JBC Papers in Press. Published on March 3, 2008 as Manuscript M709965200
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M709965200

CLASS-SPECIFIC REGULATION OF PRO-INFLAMMATORY GENES BY MYD88 PATHWAYS AND IκBζ

Hisako Kayama[1,3#], Vladimir R. Ramirez-Carrozzi[2#], Masahiro Yamamoto[1], Taketoshi Mizutani[4], Hirotaka Kuwata[3], Hideo Iba[4], Makoto Matsumoto[3], Kenya Honda[1], Stephen T. Smale[2], and Kiyoshi Takeda[1,3]

Laboratory of Immune Regulation, Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan[1], Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, California 90095-1662, USA[2], Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan[3], Department of Microbiology and Immunology, Division of Host-Parasite Interaction, Institute of Medical Science, University of Tokyo, Tokyo, Japan[4]

# These authors contributed equally to this work.

Running head; IκBζ-mediated activation of TLR-dependent genes

Address correspondence to: Kiyoshi Takeda, MD, PhD, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Fax: 81-6-6879-3989; ktakeda@ongene.med.osaka-u.ac.jp,
Stephen T. Smale, PhD, HHMI/UCLA 6525 MRL, 675 Charles E. Young Drive South, Los Angeles, USA 90095-1662, Fax: 310-206-8623, smale@mednet.ucla.edu

Toll-like receptors trigger the induction of primary response genes via MyD88-mediated activation of NF-κB and other transcription factors. These factors then act in concert with primary response gene products to induce secondary response genes. Although the MyD88 pathway is important for the expression of both primary and secondary response genes, we show that the recruitment of NF-κB, RNA polymerase, and the TATA-binding protein is MyD88-dependent only at secondary response genes. This selective dependence correlates with the fact that MyD88 is required for nucleosome remodeling and histone H3K4 trimethylation at secondary response promoters, whereas rapidly induced primary response promoters are assembled into poised MyD88-independent chromatin structures. At a subset of secondary response promoters, IκBζ was identified as a selective regulator of H3K4 trimethylation and pre-initiation complex assembly after nucleosome remodeling. These mechanistic distinctions advance our understanding of the diverse molecular cascades that underlie the differential regulation of pro-inflammatory genes.

Toll-like receptor (TLR)-dependent recognition of microbial components controls immune responses through the activation of innate immunity and the subsequent development of antigen-specific adaptive immunity (1-3). Excessive activation of innate immunity has been shown to be associated with several immune disorders (4,5). Therefore, TLR-mediated innate immune responses are finely controlled through the regulation of signaling cascades and the modulation of gene induction (5-7). TLR-mediated signaling consists of at least two pathways--a MyD88-dependent pathway and a TRIF-dependent pathway. In contrast to the selective role of TRIF in TLR3- and TLR4-mediated responses, MyD88 acts downstream of almost all TLRs to promote the activation of a broad range of pro-inflammatory and anti-microbial genes (8).

One gene that is induced in response to TLR signaling is *Nfkbiz*, which encodes a nuclear IκB family member, IκBζ (9-11). Because *Nfkbiz* expression is induced rapidly in the absence of new protein synthesis, it is considered to be a primary response gene. Newly synthesized IκBζ protein then triggers the induction of a subset of TLR-dependent secondary genes through the modulation of NF-κB activity (12). Thus, in IκBζ-deficient mice, rapidly induced primary response genes, including *Cxcl2*, *Cxcl1*, and *Il23a*, were activated normally (13). In contrast, impaired expression was observed with a subset of secondary response genes that, in wild-type mice, are induced at relatively late times after TLR stimulation, including *Il12b*, *Il6*, and *Lcn2* (13).

Although the mechanism by which IκBζ regulates secondary response genes is not known, accumulating evidence has demonstrated that chromatin structure plays a critical role in gene activation and suppression in cells of the immune system (14-18). Two main mediators, ATP-dependent nucleosome remodeling complexes and histone-modifying enzymes, help regulate chromatin structure (19-23). ATP-dependent chromatin remodeling complexes use the energy of ATP hydrolysis to disrupt histone-DNA interactions, whereas histone-modifying enzymes alter the N-terminal tails and core domains of histones to regulate the activation and suppression of transcription. Among these histone modifications, which include acetylation, methylation, ubiquitination, and sumoylation of lysine residues, methylation of specific lysine residues of histones H3 and H4 is well associated with gene activation or suppression. Of particular relevance to the current study, di- and tri-methylation of H3 Lys4 (H3K4) are generally found at genes that are competent for activation, with H3K4 trimethylation often linked to active transcription (24,25).

Initial evidence that chromatin structure may be critical for the differential regulation of primary and secondary response genes following TLR stimulation was provided in an influential study by Saccani and Natoli (26). Specifically, chromatin immunoprecipitation (ChIP) experiments revealed that NF-κB associates rapidly with rapidly induced primary response genes, but much more slowly with genes induced with delayed kinetics. Saccani and Natoli hypothesized that the association of NF-κB was delayed because changes in chromatin structure at this latter class of genes must precede NF-κB binding and transcriptional activation (26,27). More recently, this hypothesis received support from studies of the SWI/SNF family of ATP-dependent nucleosome remodeling complexes (28). SWI/SNF-dependent nucleosome remodeling was found to be important for the activation of secondary response genes and a subset of primary response genes induced with delayed kinetics. However, nucleosome remodeling by SWI/SNF complexes was not necessary for induction of rapidly induced primary response genes. Further analysis of chromatin structure using a restriction enzyme accessibility assay revealed inducible nucleosome remodeling at the promoters of secondary response and late primary response genes, with constitutively accessible chromatin observed at the promoters of early primary response genes. However, the contributions of specific signaling pathways and transcription factors to the differential regulation of primary and secondary response genes were not examined.

The importance of TLR signaling through MyD88 for the induction of a broad range of genes raises the intriguing question of whether the MyD88-dependent pathway makes similar or different contributions to the activation of primary and secondary response genes. The selective role of the primary response gene product IκBζ in regulating a subset of secondary response genes is equally intriguing, as, *a priori*, it must carry out a function that is not required for the activation of primary response genes. IκBζ could therefore be essential for nucleosome remodeling at a subset of secondary response genes or could catalyze another chromatin-related event that is not necessary for primary response gene activation.

In this study, we found that, in murine macrophages responding to LPS through TLR4, MyD88 is required for the recruitment of NF-κB p65, RNA polymerase II (pol II), and the TATA-binding protein (TBP) to secondary response promoters. However, because of redundancy with the TRIF-dependent pathway, MyD88 was not required for the recruitment of these factors to primary response promoters, although it is essential for efficient induction of primary response gene transcription. At the secondary response promoters, MyD88 was also essential for nucleosome remodeling and histone H3K4 trimethylation, whereas primary response promoters were assembled into constitutively open chromatin structures in unstimulated cells, with pre-existing H3K4 trimethylation. Surprisingly, although the function of IκBζ was restricted to secondary response genes, it was not necessary for nucleosome remodeling at these genes, but rather was important for H3K4 trimethylation and pre-initiation complex assembly downstream of the remodeling event.

Experimental procedures

Antibodies and Mice- Antibodies against NF-κB p65 (C-20) (sc-372), Pol II (H-224) (sc-9001) and TFIID (TBP) (Sl-1) (sc-273) were purchased from Santa Cruz. Antibodies to trimethyl-histone H3 (Lys4) (07-473) and SNF2β/BRG1 (07-478) were purchased from

Upstate Biotechnology. Polyclonal anti-IκBζ Ab was obtained by immunizing rabbit with a recombinant protein containing the N-terminal region of murine IκBζ (1-380 aa).

Myd88$^{-/-}$, *Trif*$^{-/-}$, and *Nfkbiz*$^{-/-}$ mice were generated as described previously (13,29). All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kyushu University and Osaka University.

Stable cell lines-RAW 264.7 cells were transfected with pcDNA 3.1 (+)-Flag-IκBζ. The cells resistant to G418 were selected in the presence of 0.4mg/ml G418 and cloned. Expression of IκBζ mRNA was determined by real-time RT-PCR and expression of Flag-IκBζ protein was monitored by Western blotting using anti- M2 monoclonal Ab (Sigma).

Cell culture- For isolation peritoneal macrophages, mice were intraperitoneally injected with 2ml of 4% thioglycollate medium (Sigma). Peritoneal exudate cells were isolated from the peritoneal cavity 3 days post injection. Cells were incubated for overnight and washed PBS. Remaining adherent cells were used as peritoneal macrophages for the experiments. To prepare bone marrow-derived macrophages, bone marrow cells were prepared from femora and passed through nylon mesh. Then, cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 μM 2ME, and 30% supernatants of cultured L cells. After 6 days, the cells were used as macrophages for experiments. Macrophage cell line RAW 264.7 cells and J774 cells were maintained in RPMI 1640 medium containing 10% FCS, 100 μM 2ME. Peritoneal macrophages, bone marrow-derived macrophages and RAW 264.7 cells were stimulated with E. coli O55:B5 LPS (Sigma).

Quantitative real-time RT-PCR- Total RNA was isolated with TRIzol reagent (Invitrogen), and 1-2 μg of RNA was reverse transcribed using M-MLV reverse transcriptase (Promega) and random primers (Toyobo) after treatment with RQ1 DNase I (Promega). Quantitative real-time PCR was performed on an ABI 7000 (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems). All data were normalized to the corresponding gene *Eef1a1* encoding elongation factor-1α (EF-1α) or 18S rRNA expression, and the fold difference relative to the EF-1α or 18S rRNA level was shown. Amplification conditions were: 50°C (2 min), 95°C (10 min), 40 cycles of 95°C (15 s), and 60°C (60 s). Primers of 18S ribosomal RNA, *Cxcl2, Il23a, Tnf, Lcn2*, and *Nfkbiz* were purchased from Assay on Demand (Applied Biosystems). Sequence for *Eef1a1, Il12b, Il6*, and *Cxcl1* are follows: *Eef1a1*probe, 5'-gcacctgagcagtgaagccagctgct-3'; forward primer, 5'-gcaaaaacgacccaccaatg-3'; reverse primer, 5'-ggcctggatggttcaggata-3'. *Il12b* probe, 5'-ctgcagggaacacatgcccacttg-3'; forward primer, 5'-gctcaggatcgctattacaat-3'; reverse primer, 5'-tcttccttaatgtcttccact3'. *Il6* probe, 5'-ccttcttgggactgatgctggtgaca-3'; forward primer, 5'-ctgcaagagacttccatccagtt-3'; reverse primer; 5'-aagtagggaaggccgtggtt-3'. *Cxcl1* probe, 5'-ttgccctcagggccccactg-3'; forward primer, 5'-caagaacatccagagcttgaaggt-3'; reverse primer, 5'-gtg gctatgacttcggtttgg-3'.

Chromatin immunoprecipitation assay- Peritoneal macrophages, bone marrow-derived macrophages, or RAW264.7 cells were stimulated with 100ng/ml LPS for the indicated periods. Chromatin was cross-linked by 1% formaldehyde at room temperature for 10 min. The cells were scraped after washed with PBS and centrifuged at 3000 rpm, and then the pellet was resuspended in SDS buffer (50 mM Tris-HCl (pH8.0), 10 mM EDTA, 0.5% SDS). Chromatin was sonicated eight times with 30 sec pulses, centrifuged at 14000 rpm to remove debris, diluted 5-fold with ChIP dilution buffer (16.7 mM Tris-HCl, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton-X) supplemented with protease inhibitor, and precleared with salmon sperm DNA/protein A agarose (Upstate). Diluted chromatin was immunoprecipitated at 4°C overnight, immune complexes were absorbed with salmon sperm DNA/protein A agarose beads, and washed one time with low salt buffer (20 mM Tris-HCl (pH8.1), 150 mM NaCl, 0.1% SDS, 1% Triton-X, 2 mM EDTA), high salt buffer (20 mM Tris-HCl (pH8.1), 500 mM NaCl, 0.1% SDS, 1% Triton-X, 2 mM EDTA), LiCl Buffer (10 mM Tris-HCl (pH8.1), 0.25 M LiCl, 1 mM EDTA, 1% deoxycolic acid, 1% NP-40), and two times with TE buffer (10 mM Tris-HCl (pH8.1), 1 mM EDTA). Immune complexes extracted in elution buffer (1% SDS, 100 mM $NaHCO_3$), was incubated for 4 h at 65°C to revert DNA-protein cross-links. Then, the DNA was extracted by incubation in proteinase K (final 50 μg/ml) buffer for 1 h at 45°C. The purified DNA was used in PCR to assess the presence of target sequences. Promoter-specific primer was designed to include NF-κB binding site. Sequence of

primers are 5'-caacagtgtacttacgcagacg-3' and 5'-ctagctgcctgcctcattctac-3' in the *Cxcl2* promoter, 5'-ctgagcactggagactctgaag-3' and 5'-gctgggatcatggtgctgtgtt-3' in the *Cxcl1* promoter, 5'-gccacttcctccaagaac-3' and 5'-tttggaaagttggggacacc-3' in the *Tnf* promoter, 5'-atccaaagccctgggaatgtc-3' and 5'-gggtagtccatcctttaccaa-3' in the *Lcn2* promoter, 5'-agtatctctgcctccttcctt-3' and 5'-gcaacactgaaaactagtgtc-3' in the *Il12b* promoter, 5'-agaagagtgctcatgcttc-3' and 5'-agctacagacatccccagtctc-3' in the *Il6* promoter, 5'-gagatggccttgcatgaggat-3' and 5'-gccagagtctcagtcttcaac-3' in the *iNOS* promoter. Chromatin immunoprecipitation using J774 cells with reduced expression of BRG1/BRM was performed essentially as described (28). In brief, J774 cells (7.5×10^5/ well) were seeded in six-well plates, and were transduced with either empty vector or BRG1/BRM shRNA vector. The BRG1/BRM sh RNA targets a conserved region between BRG1 and BRM mRNAs (TGGAGAAGCAGCAGAAGAT). The cells were infected via spin infections on consecutive days at 2500 rpm for 1.5h and at 30 °C. After the second spin infection, puromycin (3 μg/ml) selection was started. The enrichment of transduced cells was followed by flow cytometry, and RNAi-mediated depletion was monitored by western blot. For chromatin immunoprecipitation experiments, BRG1/BRM RNAi-depleted cells and control cells were stimulated and crosslinked five days after the first spin infection.

Nuclei preparation-Peritoneal macrophages or bone marrow-derived macrophages were stimulated with 10 μg/ml LPS for the indicated periods. Cells were scraped and pelleted at 1500 rpm. Cells were washed once with PBS. The cell pellet was resuspended in NP-40 lysis buffer (10 mM Tris-HCl (pH7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 0.15 mM spermine, and 0.5 mM spermidine) and incubated on ice for 5min. Nuclei were pelleted at 1000 rpm, followed by washing with RE buffer (10 mM Tris-HCl (pH7.4), 50 mM NaCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM β-Mercaptoethanol, 0.15 mM spermine, 0.5 mM spermidine).

Restriction enzyme accessibility assay-Restriction enzyme accessibility assay was performed essentially as described (30,31). Isolated cell nuclei and restriction enzyme (100U) (*Il12b* promoter and enhancer: SpeI, *Il6* promoter: AflII) were incubated for 15 min at 37 °C. Reactions were stopped by adding proteinase K buffer (100 mM Tris-HCl (pH8.5), 200 mM NaCl, 5 mM EDTA, 0.2% SDS, 100 ng/ml proteinase K), incubated overnight at 56 °C, followed by genomic DNA isolation. Purified DNA (10-15 μg) was digested to completion to generate reference cleavage products using the following restriction enzymes: KpnI and SphI for the *Il12b* promoter and enhancer, XbaI and SpeI for *Il6*. Samples were analyzed by Southern blotting with ^{32}P-labeled gene-specific probes designed at the following regions, *Il12b* promoter (+64 to +437), *Il12b* enhancer (-8711 to -9113), *Il6* promoter (-544 to -1043).

RESULTS

Different roles of MyD88 at primary and secondary response genes. To understand how the MyD88 pathway contributes to the regulation of primary and secondary response genes, we compared LPS-stimulated wild-type and *Myd88*$^{-/-}$ macrophages. In the mutant cells, the expression of a large number of primary and secondary response genes is known to be severely reduced, despite an intact TRIF-dependent pathway (1). For this study, the *Cxcl2*, *Cxcl1*, and *Tnf* genes (encoding MIP2, GRO1, and TNF-α respectively) were monitored as examples of primary response genes, which are induced rapidly (Supplementary Fig. S1A) in the absence of a requirement for new protein synthesis (based on resistance to cycloheximide [CHX]) (28). The *Lcn2*, *Il12b*, and *Il6* genes (encoding lipocalin 2, IL-12 p40, and IL-6, respectively) were used as examples of secondary response genes, which are induced with delayed kinetics (Supplementary Fig. S1B) in a CHX-sensitive manner (28). Although expression of all seven genes was greatly reduced in *Myd88*$^{-/-}$ macrophages, residual induction was observed with the four primary response genes. This induction was largely eliminated in *Myd88*$^{-/-}$*Trif*$^{-/-}$ macrophages (Supplementary Fig. S1C).

To determine how the absence of MyD88 signaling alters the cascade of events leading to transcription initiation, chromatin immunoprecipitation (ChIP) experiments were performed. At the *Lcn2* secondary response promoter, the recruitment of the NF-κB p65 subunit, pol II, and TBP was greatly reduced in

LPS-stimulated peritoneal macrophages from *Myd88*$^{-/-}$ mice, when compared to macrophages from wild-type mice (Fig. 1A). Remarkably, these same factors were recruited normally to the *Cxcl2* primary response promoter in *Myd88*$^{-/-}$ macrophages, with only a modest delay in p65 recruitment and perhaps more transient association of pol II (Fig. 1B). Consistent with previous findings (26), factor recruitment was observed at earlier time points at the primary response promoter than at the secondary response promoter. Similar results were obtained using bone marrow-derived macrophages (data not shown). Importantly, recruitment of p65, pol II, and TBP to the *Cxcl2* promoter was eliminated in *Myd88*$^{-/-}$*Trif*$^{-/-}$ macrophages (Fig. 1C), consistent with previous evidence that NF-κB activation by LPS is only modestly delayed in *Myd88*$^{-/-}$ macrophages but eliminated in *MyD88*$^{-/-}$*Trif*$^{-/-}$ macrophages (29).

These findings suggest a hypothesis in which MyD88 is essential for a change in chromatin structure at secondary response promoters that must precede the binding of NF-κB and the assembly of a transcription pre-initiation complex. However, at primary response promoters, pre-initiation complex assembly is relatively unperturbed because (1) these genes possess a poised MyD88-independent chromatin structure in unstimulated cells, and (2) the TRIF-dependent pathway can support NF-κB activation in the absence of MyD88. It is important to emphasize that *Cxcl2* transcription is severely reduced in *Myd88*$^{-/-}$ macrophages (Supplementary Fig. 1A), despite the efficient recruitment of p65, pol II, and TBP to the *Cxcl2* promoter. Possible reasons primary response genes require the MyD88 pathway for efficient induction are considered below (see Discussion).

MyD88-dependent H3K4 trimethylation and nucleosome remodeling at secondary response promoters. To test the above hypothesis, we evaluated the importance of MyD88 for the chromatin changes that accompany gene activation in LPS-stimulated macrophages. Trimethylation of histone H3K4 was first examined because of its close association with transcriptionally active genes (23,32,33). At the promoters of two representative primary response genes, *Cxcl2* and *Cxcl1*, ChIP experiments revealed constitutively high H3K4 trimethylation in unstimulated wild-type macrophages, with no significant change following LPS stimulation (Fig. 2A). These results are consistent with previous evidence that early primary response promoters possess constitutively acetylated histones and constitutively open chromatin structures (28). Importantly, similar H3K4 trimethylation levels were observed at these promoters in *Myd88*$^{-/-}$ macrophages (Fig. 2A).

In contrast to the constitutive H3K4 trimethylation observed at the primary response promoters, this modification was strongly induced in LPS-stimulated wild-type macrophages at the promoters for two representative secondary response genes, *Lcn2* and *Il12b* (Fig. 2B). Significantly, the LPS-induced H3K4 trimethylation observed at these promoters was MyD88-dependent. We further examined nucleosome remodeling at the *Il12b* promoter using a Southern blot-based restriction enzyme accessibility assay (28,31). As previously demonstrated, strong increases in restriction enzyme cleavage efficiency were observed following LPS stimulation in wild-type macrophages (Fig. 2C) (28). However, restriction enzyme cleavage was greatly reduced in *Myd88*$^{-/-}$ macrophages (Fig. 2C). Taken together, the results in Figs. 1 and 2 strongly suggest that NF-κB, TBP, and pol II cannot associate with the promoters of secondary response genes in LPS-stimulated macrophages from *Myd88*$^{-/-}$ mice because the MyD88 pathway is required for LPS-induced H3K4 trimethylation and nucleosome remodeling at these promoters. In contrast, the recruitment of NF-κB, TBP, and pol II to primary response promoters does not depend on MyD88 because these promoters are assembled into poised chromatin structures in unstimulated cells.

IκBζ mediates the activation of a subset of secondary response genes. The above results suggest that, although the TRIF pathway can support NF-κB activation in LPS-stimulated macrophages from *Myd88*$^{-/-}$ mice, TRIF cannot support the activation of one or more factors that act prior to nucleosome remodeling and H3K4 trimethylation at MyD88-dependent secondary response promoters. Thus, a MyD88-specific target is essential for nucleosome remodeling and H3K4 trimethylation at these promoters. It is noteworthy that LPS-induced remodeling at a collection of secondary response promoters was previously found to require new protein synthesis (28). This previous finding suggests that the MyD88 target of interest may be a

primary response gene product, as opposed to a transcription factor whose activity is induced post-translationally in response to TLR4 signaling.

One primary response gene product that is an attractive candidate for contributing to the activation of a subset of secondary response genes is the nuclear IκB protein, IκBζ, encoded by the *Nfkbiz* gene. As described previously, expression of a subset of secondary response genes is impaired in *Nfkbiz*$^{-/-}$ macrophages, whereas primary response genes are expressed normally (Supplementary Fig. S2A, B) (13).

To gain further insight into the importance of IκBζ for the expression of secondary response genes, IκBζ was constitutively overexpressed in the RAW264.7 macrophage line. When IκBζ was present at the time of LPS stimulation, three IκBζ-dependent secondary response genes, *Lcn2*, *Il12b*, and *Il6*, were induced more rapidly than in control RAW264.7 cells (Supplementary Fig. S2C). Consistent with the more rapid induction in the presence of constitutively expressed IκBζ, ChIP assays revealed that the association of p65, pol II, and TBP reached a detectable level at the *Lcn2* promoter more rapidly than in control cells (Fig. 3A). In contrast, the kinetics of factor recruitment to the *Cxcl2* primary response promoter was unchanged (Fig. 3B). Importantly, histone H3K4 trimethylation was also induced more rapidly at the *Lcn2* promoter following LPS stimulation of the IκBζ-expressing cells (Fig. 3C), whereas the constitutive H3K4 trimethylation observed at the *Cxcl2* promoter remained unchanged (Fig. 3D). These findings are consistent with a model in which IκBζ plays a major role in the changes in chromatin structure that are associated with the induction of IκBζ-dependent secondary response genes.

IκBζ-dependent H3K4 trimethylation at secondary response promoters. To complement the IκBζ gain-of-function experiments, loss of function experiments were performed with bone marrow-derived macrophages from *Nfkbiz*$^{-/-}$ mice. Strikingly, although the constitutive H3K4 trimethylation at three representative primary response promoters was comparable in wild-type and *Nfkbiz*$^{-/-}$ macrophages (*Cxcl2, Cxcl1*, Fig. 4A; *Tnf*, data not shown), the inducible H3K4 trimethylation observed in wild-type macrophages at the promoters of three IκBζ-dependent secondary response promoters was greatly reduced in *Nfkbiz*$^{-/-}$ cells (*Lcn2, Il12b*; Fig. 4B, *Il6*; data not shown). Furthermore, the recruitment of NF-κB p65, pol II and TBP was greatly diminished at secondary response promoters in *Nfkbiz*$^{-/-}$ cells (*Lcn2, Il12b*; Fig. 4D, *Il6*; Supplementary Fig. S3), whereas the recruitment of these proteins to promoters of primary response or IkBz-independent secondary response genes was unaffected (*Cxcl2, Tnf*, or *iNOS* Fig. 4C and Supplementary Fig. S4). These results are consistent with the gain-of-function results and support the view that IκBζ is a selective major regulator of chromatin structure and pre-initiation complex assembly at IκBζ-dependent secondary response genes.

To determine whether IκBζ directly regulates IκBζ-dependent genes, ChIP experiments were performed. The results revealed that IκBζ associates with the *Il12b* and *Il6* promoters in LPS-stimulated macrophages (Fig. 5). The kinetics of binding was similar to that observed with two other factors previously shown to associate with these control regions, BRG1 and C/EBPβ (28). It is important to note that, in addition to its association with the promoters of IκBζ-dependent genes, inducible IκBζ association was observed in our hands at a recently described enhancer for the *Il12b* gene (Fig. 5), as well as at the promoters for a number of primary response genes that do not require IκBζ for expression (data not shown). Thus, although the analysis of *Nfkbiz*$^{-/-}$ macrophages provides strong evidence that IκBζ is selectively required for H3K4 trimethylation and for the recruitment of p65, TBP, and pol II to a subset of IκBζ-dependent secondary response genes, IκBζ associates with other LPS-induced genes with no apparent functional consequences.

IκBζ-independent nucleosome remodeling at secondary response promoters. As shown previously (28) and in Fig. 2C, nucleosome remodeling by ATP-dependent remodeling complexes is generally required for the activation of secondary response genes following LPS stimulation. To determine whether IκBζ is required for nucleosome remodeling at IκBζ-dependent genes, we first used a ChIP assay to monitor recruitment of the BRG1 catalytic subunit of the SWI/SNF remodeling complexes. Following LPS stimulation, BRG1 was found to associate with representative IκBζ-dependent genes (*Il6* and *Lcn2* in Fig. 6A and *Il6* and *Il12b* in Fig. 6B). Interestingly, this inducible association was

eliminated in *Myd88*$^{-/-}$ macrophages, but was retained in *Nfkbiz*$^{-/-}$ macrophages (Fig. 6). These results suggest that IκBζ acts downstream of the remodeling event, with another MyD88 target required for remodeling. In contrast to the results obtained with secondary response genes, BRG1 associated constitutively with the *Cxcl2* primary response promoter (Fig. 6), as previously described, even though BRG1 is not important for the induction of this and other primary response genes (28). This constitutive association was retained in both *Myd88*$^{-/-}$ and *Nfkbiz*$^{-/-}$ cells (Fig. 6).

To further evaluate the role of IκBζ in nucleosome remodeling, restriction enzyme accessibility experiments were performed. Consistent with the BRG1 ChIP data, the LPS-induced increases in restriction enzyme cleavage observed at the *Il12b* enhancer, *Il12b* promoter, and *Il6* promoter, were comparable in wild-type and *Nfkbiz*$^{-/-}$ macrophages stimulated with LPS (Fig. 7A). Finally, to determine whether IκBζ association with secondary response genes requires nucleosome remodeling, BRG1 and the closely related BRM catalytic subunits of the SWI/SNF remodeling complexes were depleted from J774 macrophages using a retrovirus that expresses an siRNA targeted to a conserved region of BRG1 and BRM (28). In cells with reduced BRG1/BRM expression, LPS-induced recruitment of IκBζ to the *Il6* and *Lcn2* promoters and the *Il12b* enhancer was reduced compared with control cells, whereas recruitment to the constitutively open *Cxcl2* and *Tnf* promoters was unchanged (Fig. 7B). These findings indicate that nucleosome remodeling is required for the efficient recruitment of IκBζ to the transcriptional control regions of secondary response genes.

DISCUSSION

The results described in this study highlight the diverse mechanisms by which chromatin structure, signal transduction pathways, and transcription factors can control the activation of a large panel of inducible pro-inflammatory genes expressed by macrophages following TLR4 stimulation. At one key class of genes that is induced rapidly in the absence of new protein synthesis – the early primary response class – the promoters appear to be assembled into chromatin structures that are poised for activation. In unstimulated cells, these chromatin structures consist of high histone acetylation and H3K4 trimethylation levels and high accessibility to nuclease cleavage (26,28). After macrophage activation, the chromatin structure remains largely unchanged, but NF-κB rapidly associates with the promoters and presumably contributes to the rapid assembly of a pre-initiation complex containing TBP and pol II. Pre-initiation complex assembly in response to TLR4 signaling does not specifically require MyD88 because of its redundancy with TRIF. However, MyD88 is critical for the efficient induction of primary response gene transcription. It is possible that reduced expression of primary response genes in *Myd88*$^{-/-}$ cells is due to earlier shut-down of transcription. But there might be another unknown mechanism, since the MyD88 effect is just as strong at the 1 hr time point.

Although secondary response genes can be induced quite rapidly after TLR4 stimulation, their mechanism of activation is dramatically different. In unstimulated macrophages, secondary response promoters are usually assembled into chromatin structures that are inaccessible to nuclease cleavage and exhibit low levels of histone acetylation and H3K4 trimethylation (26,28). Substantial changes in chromatin structure are therefore required for transcriptional activation (27). One critical event appears to be the remodeling of nucleosomes by ATP-dependent nucleosome remodeling complexes of the SWI/SNF family. A previous study showed that nucleosome remodeling at the promoters of secondary response genes requires new protein synthesis (28). The results of the current study show that MyD88 pathways are also required for nucleosome remodeling. Although IκBζ was an attractive candidate for a MyD88-dependent primary response gene product that might drive nucleosome remodeling at a subset of secondary response genes, our results strongly suggest that other MyD88-dependent primary response gene products carry out this critical function. IκBζ instead plays an important role downstream of the nucleosome remodeling step, but prior to the binding of NF-κB p65, TBP, and pol II, and prior to histone H3K4 trimethylation (Fig. 8).

The precise physiological reasons for the evolution of these diverse pro-inflammatory gene activation pathways are not known. However, one important consequence is the capacity to regulate different subsets of genes with greater selectivity. Indeed, many of the primary response gene products may function

properly only if activated rapidly and if produced at an appropriate level. For example, TNF-α and IL-1β are cytokines that trigger a series of inflammatory and host defense responses, and when produced in excess, they induce serious multiple organ failure (34). Other primary response gene products, the MIP-2 and GRO1 chemokines, mediate the recruitment of neutrophils during an acute phase of inflammation (35).

The rapid induction of many early primary response genes may therefore be of considerable benefit during the initiation of an immune response. At the same time, the need to overcome a nucleosome barrier may provide important benefits for secondary response genes, by allowing them to be activated with a higher degree of selectivity by different stimuli and in different biological scenarios. Consistent with this hypothesis, only a subset of secondary response genes require IκBζ for activation, whereas other transcription factors presumably carry out the same functions at other subsets of secondary response genes. In fact, our results provide evidence that the nucleosome barrier can confer a requirement for at least two transcription factors that are not generally required by early primary response genes. That is, at least one MyD88 target gene must be required for inducible nucleosome remodeling by SWI/SNF complexes, with IκBζ or its equivalent functioning at a later stage of the gene activation cascade to promote pre-initiation complex assembly and histone H3K4 trimethylation. In this context, it is interesting to note that IκBζ-dependent secondary response gene products include cytokines and chemokines that are involved in the regulation of T cell-mediated immune responses: IL-12 p40 is a key subunit of the IL-12 and IL-23 heterodimeric cytokines, which are critical to Th1 and Th17 development, respectively (36), and IL-6 has recently been shown to be essential for initiation of Th17 cell development (37). Other IκBζ-dependent secondary response gene products, such as Ebi3, IL-18 and TARC (13), also regulate Th1/Th2/Th17 cell-mediated immune responses (38-42). Therefore, the nucleosome barrier for secondary response genes may have evolved to ensure tight regulation of adaptive immunity during TLR signaling.

Although the results of this analysis provide a framework toward understanding the differential regulation of pro-inflammatory genes, a number of important mechanistic questions remain to be answered. First, why is MyD88 required for primary response gene activation, despite the efficient recruitment of p65, TBP, and pol II to primary response promoters in *Myd88*$^{-/-}$ macrophages? A likely explanation is that these genes require a direct target of the MyD88 signaling pathway that remains to be characterized. However, an alternative is that the moderately delayed induction of NF-κB somehow disrupts transcriptional activation.

A second unanswered question is, what primary response gene products and MyD88 target genes are responsible for inducible remodeling at IκBζ-dependent secondary response genes, as well as other subsets of secondary response genes? This question has been especially difficult to answer. IκBζ appeared to be an ideal candidate because its regulatory functions are restricted to secondary response genes. However, we were unable to find evidence implicating IκBζ in the regulation of nucleosome remodeling. Analyses of several other primary response gene products for a possible role in the regulation of nucleosome remodeling at secondary response genes have also yielded negative results (V.R.C. and S.T.S., unpublished data). As an alternative to the candidate-gene approach, it may be possible to identify DNA sequence elements in secondary response promoters that are required for inducible nucleosome remodeling, which may lead to the critical transcription factors. However, it will first be necessary to develop an assay in which secondary response promoters assemble into a native chromatin structure that depends on a nucleosome remodeling event for transcriptional activation.

A third unanswered question is how IκBζ contributes to the recruitment of NF-κB p65 complexes, TBP, and pol II, and how it facilitates histone H3K4 trimethylation. A previous study provided evidence that IκBζ is recruited to target promoters by NF-κB p50 homodimers (13). One possibility is that the binding of p50 homodimers recruits IκBζ through a direct interaction, which then recruits a p65-containing dimer to a different NF-κB site, or to the same site through dimer exchange. The p65 dimer could then act in concert with other transcription factors bound to the promoter to recruit TBP and pol II and facilitate H3K4 trimethylation and transcription initiation. Other possible mechanisms of IκBζ

function must also be considered, such as a direct role in the recruitment of an H3K4 methyltransferase.

The importance of chromatin for the differential regulation of TLR-dependent genes was recently highlighted in an elegant analysis of the negative regulation of the inflammatory response (18). Further dissection of the diverse mechanisms underlying these key regulatory events will help elucidate the molecular basis of immune disorders caused by abnormal activation of innate immunity.

REFERENCES

1. Akira, S., and Takeda, K. (2004) *Nat Rev Immunol* **4**(7), 499-511
2. Iwasaki, A., and Medzhitov, R. (2004) *Nat Immunol* **5**(10), 987-995
3. Beutler, B., Jiang, Z., Georgel, P., Crozat, K., Croker, B., Rutschmann, S., Du, X., and Hoebe, K. (2006) *Annu Rev Immunol* **24**, 353-389
4. Marshak-Rothstein, A. (2006) *Nat Rev Immunol* **6**(11), 823-835
5. Liew, F. Y., Xu, D., Brint, E. K., and O'Neill, L. A. (2005) *Nat Rev Immunol* **5**(6), 446-458
6. Negishi, H., Ohba, Y., Yanai, H., Takaoka, A., Honma, K., Yui, K., Matsuyama, T., Taniguchi, T., and Honda, K. (2005) *Proc Natl Acad Sci U S A* **102**(44), 15989-15994
7. Kuwata, H., Matsumoto, M., Atarashi, K., Morishita, H., Hirotani, T., Koga, R., and Takeda, K. (2006) *Immunity* **24**(1), 41-51
8. Akira, S., Uematsu, S., and Takeuchi, O. (2006) *Cell* **124**(4), 783-801
9. Yamazaki, S., Muta, T., and Takeshige, K. (2001) *J Biol Chem* **276**(29), 27657-27662
10. Kitamura, H., Kanehira, K., Okita, K., Morimatsu, M., and Saito, M. (2000) *FEBS Lett* **485**(1), 53-56
11. Haruta, H., Kato, A., and Todokoro, K. (2001) *J Biol Chem* **276**(16), 12485-12488
12. Motoyama, M., Yamazaki, S., Eto-Kimura, A., Takeshige, K., and Muta, T. (2005) *J Biol Chem* **280**(9), 7444-7451
13. Yamamoto, M., Yamazaki, S., Uematsu, S., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Kuwata, H., Takeuchi, O., Takeshige, K., Saitoh, T., Yamaoka, S., Yamamoto, N., Yamamoto, S., Muta, T., Takeda, K., and Akira, S. (2004) *Nature* **430**(6996), 218-222
14. Smale, S. T., and Fisher, A. G. (2002) *Annu Rev Immunol* **20**, 427-462
15. Ansel, K. M., Lee, D. U., and Rao, A. (2003) *Nat Immunol* **4**(7), 616-623
16. Mostoslavsky, R., Alt, F. W., and Bassing, C. H. (2003) *Nat Immunol* **4**(7), 603-606
17. Natoli, G., Saccani, S., Bosisio, D., and Marazzi, I. (2005) *Nat Immunol* **6**(5), 439-445
18. Foster, S. L., Hargreaves, D. C., and Medzhitov, R. (2007) *Nature* **447**(7147), 972-978
19. Roth, S. Y., Denu, J. M., and Allis, C. D. (2001) *Annu Rev Biochem* **70**, 81-120
20. Lusser, A., and Kadonaga, J. T. (2003) *Bioessays* **25**(12), 1192-1200
21. Cairns, B. R. (2005) *Curr Opin Genet Dev* **15**(2), 185-190
22. de la Serna, I. L., Ohkawa, Y., and Imbalzano, A. N. (2006) *Nat Rev Genet* **7**(6), 461-473
23. Li, B., Carey, M., and Workman, J. L. (2007) *Cell* **128**(4), 707-719
24. Bernstein, B. E., Humphrey, E. L., Erlich, R. L., Schneider, R., Bouman, P., Liu, J. S., Kouzarides, T., and Schreiber, S. L. (2002) *Proc Natl Acad Sci U S A* **99**(13), 8695-8700
25. Martin, C., and Zhang, Y. (2005) *Nat Rev Mol Cell Biol* **6**(11), 838-849
26. Saccani, S., Pantano, S., and Natoli, G. (2001) *J Exp Med* **193**(12), 1351-1359
27. Natoli, G. (2006) *FEBS Lett* **580**(12), 2843-2849
28. Ramirez-Carrozzi, V. R., Nazarian, A. A., Li, C. C., Gore, S. L., Sridharan, R., Imbalzano, A. N., and Smale, S. T. (2006) *Genes Dev* **20**(3), 282-296
29. Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S. (2003) *Science* **301**(5633), 640-643
30. Weinmann, A. S., Plevy, S. E., and Smale, S. T. (1999) *Immunity* **11**(6), 665-675
31. Zhou, L., Nazarian, A. A., and Smale, S. T. (2004) *Mol Cell Biol* **24**(6), 2385-2396
32. Santos-Rosa, H., Schneider, R., Bannister, A. J., Sherriff, J., Bernstein, B. E., Emre, N. C., Schreiber, S. L., Mellor, J., and Kouzarides, T. (2002) *Nature* **419**(6905), 407-411
33. Bernstein, B. E., Kamal, M., Lindblad-Toh, K., Bekiranov, S., Bailey, D. K., Huebert, D. J., McMahon, S., Karlsson, E. K., Kulbokas, E. J., 3rd, Gingeras, T. R., Schreiber, S. L., and Lander, E. S. (2005) *Cell* **120**(2), 169-181
34. Dinarello, C. A. (1991) *J Infect Dis* **163**(6), 1177-1184
35. Zhang, X. W., Liu, Q., Wang, Y., and Thorlacius, H. (2001) *Br J Pharmacol* **133**(3),

413-421
36. Trinchieri, G., Pflanz, S., and Kastelein, R. A. (2003) *Immunity* **19**(5), 641-644
37. Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L., and Kuchroo, V. K. (2006) *Nature* **441**(7090), 235-238
38. Hunter, C. A. (2005) *Nat Rev Immunol* **5**(7), 521-531
39. Batten, M., Li, J., Yi, S., Kljavin, N. M., Danilenko, D. M., Lucas, S., Lee, J., de Sauvage, F. J., and Ghilardi, N. (2006) *Nat Immunol* **7**(9), 929-936
40. Stumhofer, J. S., Laurence, A., Wilson, E. H., Huang, E., Tato, C. M., Johnson, L. M., Villarino, A. V., Huang, Q., Yoshimura, A., Sehy, D., Saris, C. J., O'Shea, J. J., Hennighausen, L., Ernst, M., and Hunter, C. A. (2006) *Nat Immunol* **7**(9), 937-945
41. Nakanishi, K., Yoshimoto, T., Tsutsui, H., and Okamura, H. (2001) *Annu Rev Immunol* **19**, 423-474
42. Imai, T., Nagira, M., Takagi, S., Kakizaki, M., Nishimura, M., Wang, J., Gray, P. W., Matsushima, K., and Yoshie, O. (1999) *Int Immunol* **11**(1), 81-88

FOOTNOTES

We thank Y. Yamada and K. Takeda for technical assistance, T. Nakayama for helpful discussion, and M. Kurata and M. Yasuda for secretarial assistance. This work was supported by Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare, as well as the Tokyo Biochemical Research Foundation, the Cell Science Research Foundation, the Yakult Bio-Science Foundation, the Osaka Foundation for Promotion of Clinical Immunology, the Sumitomo Foundation, and the Sankyo Foundation of Life Science. Additional support was provided by the Giannini Family Foundation (V.R.R.C.) and the Howard Hughes Medical Institute (S.T.S.).

Figure legends

Fig. 1. MyD88-dependent regulation of primary and secondary response genes.
Peritoneal macrophages from wild-type, *Myd88*$^{-/-}$ and *Myd88*$^{-/-}$*Trif*$^{-/-}$ mice were stimulated with 100 ng/ml LPS for the indicated periods, and chromatin immunoprecipitation (ChIP) assay was performed with antibodies to NF-κBp65, pol II, or TBP. The immunoprecipitated *Lcn2* promoter (A) or *Cxcl2* promoter (B, C) was analyzed by PCR with promoter-specific primers. PCR amplification of the total input DNA in each sample is shown (Input). Representative of five independent experiments. The same result was obtained when bone marrow-derived macrophages were used.

Fig. 2. MyD88-dependent nucleosome remodeling of secondary response promoters.
Peritoneal macrophages from wild-type and *Myd88*$^{-/-}$ mice were stimulated with 100 ng/ml LPS for the indicated periods, and chromatin immunoprecipitation (ChIP) assay was performed with anti-trimethyl-Histone H3 (Lys4) Ab (Me3-H3K4). Precipitated DNA for the *Cxcl2* promoter, *Cxcl1* promoter (A), *Lcn2* promoter or *Il12b* promoter (B) was analyzed by PCR. Representative of two independent experiments. (C) Bone marrow macrophages from wild type and Myd88$^{-/-}$ mice were stimulated with 10 μg/ml LPS for the indicated periods. Restriction enzyme accessibility assay at *Il12b* promoter region using nuclei from bone marrow macrophages.

Fig. 3. IκBζ-mediated activation of secondary response gene promoters. RAW264.7 cells stably expressing IκBζ were treated with 100 ng/ml LPS for the indicated periods, then used for ChIP assay with antibodies to NF-κBp65, pol II, or TBP. (A, B), or anti-trimethyl-H3 (Lys4) Ab (C, D). The immunoprecipitated *Lcn2* promoter (A, C) or *Cxcl2* promoter (B, D) was analyzed by PCR with promoter-specific primers.

Fig. 4. Impaired trimethylation of histone H3 Lys4 and pre-initiation complex assembly at

secondary response promoters in *Nfkbiz*$^{-/-}$ macrophages. (A, B) Chromatin prepared from wild-type and *Nfkbiz*$^{-/-}$ bone marrow-derived macrophages treated with 100 ng/ml LPS for the indicated periods was immunoprecipitated with antibody against trimethyl-histone H3 (Lys4). Precipitated DNA was analyzed by PCR with promoter-specific primers for *Cxcl2*, *Cxcl1* (A), *Lcn2* , and *Il12b* (B). Representative of three independent experiments. (C, D) Peritoneal macrophages from wild-type and *Nfkbiz*$^{-/-}$ mice were stimulated with 100 ng/ml LPS for the indicated periods, then chromatins were prepared and immunoprecipitated with antibody against NF-κBp65, pol II, or TBP. Precipitated DNA was analyzed by PCR with promoter-specific primers for *Cxcl2*, *Tnf* (C), *Lcn2*, and *Il12b* (D). The results are representative of three independent experiments.

Fig. 5. Recruitment of IκBζ and BRG1 to the same control regions of the *Il12b* and *Il6* loci. J774 cells were stimulated with LPS for 0, 30, and 240 min, and chromatins were prepared and precipitated with antibodies against BRG1, IκBζ and C/EBPβ. Precipitated DNA samples were amplified using primer pairs specific to the indicated regions relative to the *Il12b* and *Il6* transcriptional start site.

Fig. 6. MyD88-dependent, but IκBζ-independent, recruitment of BRG1 to secondary response promoters. Bone marrow-derived macrophages from wild-type, *Myd88*$^{-/-}$ (A) and *Nfkbiz*$^{-/-}$ (B) mice were stimulated with 100 ng/ml LPS for the indicated periods. Then, chromatins were prepared and precipitated with anti-BRG1 Ab. Precipitated DNA samples were amplified using promoter-specific primers for *Cxcl2* (A, B), *Il6* (A, B), *Lcn2* (A), and *Il12b* (B).

Fig. 7. Nucleosome remodeling at secondary response gene regulatory regions in the absence of IκBζ. (A) Bone marrow-derived macrophages from wild-type and *Nfkbiz*$^{-/-}$ mice were stimulated with 10 μg/ml LPS for the indicated periods. Restriction enzyme accessibility assay at the *Il12b* enhancer (upper), *Il12b* promoter (middle), *Il6* promoter (bottom) regions were performed. (B) ChIP assay was performed with chromatin prepared from J774 cells infected with the empty RNAi vector (white bars) and from BRG1/BRM siRNA-depleted cells (black bars) treated with LPS for 0 and 240 min. Antibodies against IκBζ and BRG1 were used. Precipitated DNA was quantified by real-time PCR using primers specific for the indicated control regions. Data were plotted relative to input DNA (% Input).

Fig. 8. Schematic model of primary and secondary response gene activation pathway.
Early primary response gene promoters have open nucleosome structures and are activated immediately after LPS stimulation. In contrast, secondary response gene promoters have closed nucleosome structures, and are remodeled through an unknown primary response gene product (X)-dependent recruitment of the SWI/SNF complexes, including BRG1. Then, another primary response gene product, IκBζ, mediates pre-initiation complex assembly and histone H3K4 trimethylation, resulting in activation of the secondary response genes.

Figure 1

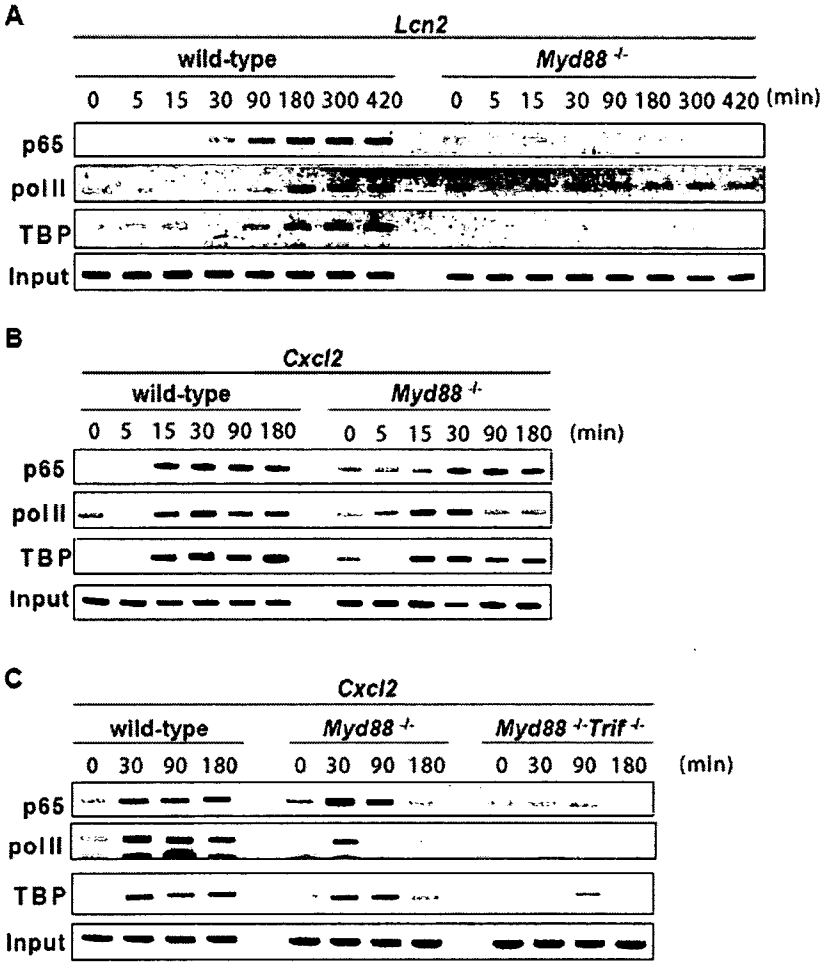
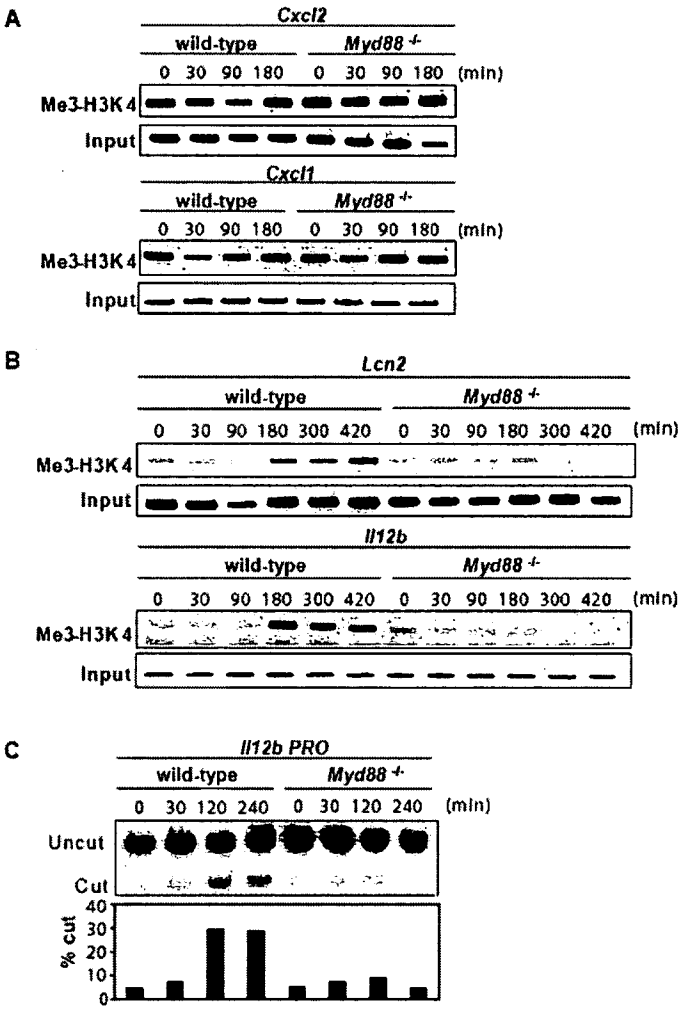

A
Lcn2
wild-type
Myd88 -/-
0 5 15 30 90 180 300 420 0 5 15 30 90 180 300 420 (min)
p65
polII
TBP
Input
B
Cxcl2
wild-type
Myd88 -/-
0 5 15 30 90 180 0 5 15 30 90 180 (min)
p65
polII
TBP
Input
C
Cxcl2
wild-type
Myd88 -/-
Myd88 -/-Trif -/-
0 30 90 180 0 30 90 180 0 30 90 180 (min)
p65
polII
TBP
Input

Figure 2


A
Cxcl2
wild-type
Myd88 -/-
0 30 90 180 0 30 90 180 (min)
Me3-H3K4
Input
Cxcl1
wild-type
Myd88 -/-
0 30 90 180 0 30 90 180 (min)
Me3-H3K4
Input
B
Lcn2
wild-type
Myd88 -/-
0 30 90 180 300 420 0 30 90 180 300 420 (min)
Me3-H3K4
Input
Il12b
wild-type
Myd88 -/-
0 30 90 180 300 420 0 30 90 180 300 420 (min)
Me3-H3K4
Input
C
Il12b PRO
wild-type
Myd88 -/-
0 30 120 240 0 30 120 240 (min)
Uncut
Cut
% cut
40
30
20
10
0