core protein on ER may positively support virus production under the fulminant condition.

REGULATION OF HOST LIPID METABOLISM BY THE CORE PROTEIN

The mechanisms by which the core protein can induce liver diseases and extrahepatic manifestations are unknown. Liver steatosis, which is one of the characteristics associated with persistent HCV infection, develops by accumulation of triglyceride-rich lipids in hepatocytes. However, the precise functions of HCV proteins in the development of fatty liver remain unknown due to the lack of an adequate system to investigate the pathogenesis of HCV. HCV core protein expression has been shown to induce lipid droplets in cell lines and hepatic steatosis and hepatocellular carcinoma in transgenic mice (Barba et al., 1997; Moriya et al., 1997; Hope and McLauchlan, 2000). The lipid composition of the core-transgenic mouse is similar to that of a hepatitis C patient (Koike et al., 2010; Miyoshi et al., 2011). These reports suggest that HCV core protein plays an important role in the development of various types of liver failure, including steatosis and hepatocellular carcinoma. Biosynthesis of triglycerides is mainly regulated by the sterol regulatory element-binding protein (SREBP)-1c. It has been reported that many genes regulated by SREBPs were induced during the early stage of HCV infection in the livers of chimpanzees (Bigger et al., 2004). Our study has demonstrated that the core protein enhances the binding activity of the LXRα–RXRα complex to the *srebp-1c* promoter in a PA28γ-dependent manner, resulting in upregulation of SREBP-1c and its regulating genes (Moriishi et al., 2007). The activation may be mediated by the direct interaction between the core protein and RXRα (Tsutsumi et al., 2002). Another mechanism is thought to be suppression of lipid secretion. Reduced serum levels of cholesterol and apolipoprotein B have been reported in patients with severe hepatitis C and core-transgenic mice (Perlemuter et al., 2002). The MTP regulates the assembly and secretion of VLDLs consisting of apolipoprotein E, cholesterol, and triglycerides. In core-transgenic mice, MTP-specific activity is significantly decreased (Perlemuter et al., 2002). In addition, DGAT1, which plays an important role in trafficking core protein from lipid droplets to the ER membrane (Herker et al., 2010), was reported to delay the turnover of lipid droplets that are coated by the core protein (Harris et al., 2011; **Figure 3**). Furthermore, increases in saturated and monounsaturated fatty acids enhance HCV RNA replication (Kapadia and Chisari, 2005). The core protein can enhance the production of reactive oxygen species (ROS) by induction of induced nitric oxide synthetase (iNOS) or by damage to the mitochondrial electron transport system, contributing to the emergence of hepatocellular carcinoma (Moriya et al., 2001; Okuda et al., 2002; Nunez et al., 2004), suggesting that accumulation of lipids hastens the occurrence of hepatocellular carcinoma by enhancing ROS production. The core protein is reported to be degraded by PA28gamma-dependent, but ubiquitin-independent, proteasome activity, and directly binds to PA28gamma (Moriishi et al., 2003; Suzuki et al., 2009). PA28gamma knockdown diminished liver steatosis, hepatocellular carcinoma, and insulin resistance induced by HCV core protein in the mouse liver (Moriishi et al., 2007). After our reports, several groups found that PA28gamma plays an important role in cell cycling by degradation of SRC-3, p16, p19, and p53 (Li et al., 2006; Chen et al., 2007; Zhang and Zhang, 2008). Furthermore, HCV propagation in a cell culture system is potently suppressed by PA28gamma knockdown, regardless of cell growth (Moriishi et al., 2010). One possibility is that E6AP-dependent ubiquitination of the core protein in cytoplasm is competitively suppressed by peptide fragments deduced from nuclear core protein. However, there is still the possibility of an indirect effect of PA28gamma, since potent reduction of PA28gamma, but not intermediate reduction, can induce nuclear accumulation of HCV core protein in cultured cells and the mouse liver, but both potent and intermediate reductions could suppress viral production (Moriishi et al., 2007, 2010; Cerutti et al., 2011). Further study will be required to clarify the mechanism by which PA28gamma regulates core-induced liver diseases and the HCV life cycle.

NS3/4A AND LIPID DROPLETS

The NS3 also cleaves the host adaptor proteins IPS-1/MAVS and TRIF to modulate TLR and RIG-I signaling, resulting in inhibition of type I interferon production (Ferreon et al., 2005; Li et al., 2005a,b; Cheng et al., 2006; Loo et al., 2006). It is speculated that NS3 suppresses the activation of host innate immunity induced by HCV RNA and then contributes to persistent infection with HCV. NS3/4A may be responsible for not only the replication, but also the virus assembly and production by interaction with viral and host proteins on a region close to lipid droplets/ER assembly site. NS3/4A interacts with NS2 cooperating with p7 and E2 to recruit the core protein from lipid droplets to the cytoplasmic motile puncta along microtubules (Boson et al., 2011; Counihan et al., 2011; Popescu et al., 2011). HCV NS3/4A also interacts with host protein Y-box-binding protein-1 (YB-1) and influences the equilibrium between viral replication and infectious particle production (Chatel-Chaix et al., 2011). Knockdown of YB-1 impaired HCV RNA replication, regardless of the viral genotype, but did not affect NS3/4A autoprocessing and MAVS cleavage (Chatel-Chaix et al., 2011). JFH1 infection allowed YB-1 to translocate to lipid droplets containing core protein and NS3 (Chatel-Chaix et al., 2011), although knockdown of YB-1 enhanced the production of viral infectious particles (Chatel-Chaix et al., 2011). YB-1 may cooperate with NS3/4A to negatively regulate the steps after replication and to positively regulate viral replication.

NS5A AND CYCLOPHILINS

The peptide bond *cis/trans* isomerase converts between *cis* and *trans* peptide bonds leading to correct folding of the protein substrate. Peptidyl prolyl *cis/trans* isomerase (PPIase) includes the families of cyclophilin (Fischer et al., 1989), FK506-binding proteins (FKBP; Siekierka et al., 1989a,b) and parvulins (Rahfeld et al., 1994), and the secondary amide peptide bond *cis/trans* isomerase (Schiene-Fischer et al., 2002). Cyclophilin and FKBP are categorized as immunophilins, which are targeted by the immunosuppressants cyclosporin and FK506, respectively (Liu et al., 1991). Some cyclophilins and FKBP8 were shown to interact with NS5B and/or NS5A and to regulate HCV replication (Watashi et al., 2005; Okamoto et al., 2006), suggesting that immunophilins could lead to promising therapies for chronic hepatitis C, as discussed below.

Cyclosporin A and its derivatives, which target cyclophilins, were shown to impair HCV RNA replication and to exhibit efficacy in hepatitis C patients (Watashi et al., 2003; Ishii et al., 2006). Inoue et al. (2003) reported cyclosporin A treatment of HCV in a clinical trial. Cyclosporin derivatives lacking the ability to interact with cyclophilin lost their inhibitory effect on HCV replication (Watashi et al., 2005). Cyclophilin B is reported to be a 20-kDa secreted neurotropic factor for spinal cord cells in chick embryos (Spik et al., 1991), and is secreted into human milk and blood (Spik et al., 1991; Allain et al., 1994). Cyclophilin B specifically interacts with NS5B, the HCV RNA-dependent RNA polymerase around the ER of the HCV replicon cells, and promotes NS5B's association with viral RNA (Watashi et al., 2005). Cyclosporin A (CsA) was shown to disrupt the interaction between NS5B and cyclophilin B (Watashi et al., 2005). Treatment with cyclosporin A and knockdown of cyclophilin B suppressed the replication of HCV. However, several groups reported that interaction between NS5A and cyclophilin A is more important for HCV replication than interaction between NS5B and cyclophilin B. There is a growing consensus that cyclophilin A in particular is a crucial factor during HCV replication. A number of point mutations in both NS5A and NS5B have been reported to be associated with *in vitro* resistance to cyclophilin A (Yang et al., 2008; Chatterji et al., 2009; Kaul et al., 2009). Direct interaction between cyclophilin A and NS5B or NS5A has been observed (Yang et al., 2008). Several CsA-analogs, i.e., NIM811 (Ma et al., 2006), DEB025, and SCY-635 (Hopkins et al., 2010), are currently in preclinical and clinical development. DEB025 disrupts the interaction between NS5A and cyclophilin A and suppresses cyclophilin A isomerase activity. Although experimental differences in cell lines and replicons may affect employment of cyclophilins in HCV replication, the main molecule targeted by the cyclosporin analogs used clinically so far seems to be cyclophilin A.

The treatment with CsA has been associated with increased susceptibility to atherosclerosis and the development of hyperlipidemia (reviewed by Kockx et al., 2010). Treatment with CsA upregulated activity of cholesteryl ester transfer protein and suppressed lipoprotein lipase activity (Tory et al., 2008). Upregulation of cholesteryl ester by cholesteryl ester transfer protein could lead to accumulation of lipoprotein with cholesteryl ester. The report by Anderson et al. (2011) suggests that cyclophilin A and cyclophilin 40 are important for not only viral replication, but also the release of infectious viral particles. NIM811 treatment suppresses virus production and viral RNA replication (Goto et al., 2006). NIM811 treatment led to enlargement of lipid droplets and apoB crescent formation in replicon cells, but not naïve Huh7 cell line, while decreasing apoB secretion and the number of lipid droplets, rendering NS5A dislocation with apoB (Anderson et al., 2011). Knockdown of cyclophilins A and 40 in replicon cells showed the similar changes in lipid droplets size and apoB localization, comparing with NIM811 treatment (Anderson et al., 2011). Cyclophilins A and 40 may regulate lipid trafficking in the presence of HCV proteins to support secretion of viral particles.

NS5A/B AND MEMBRANE-ASSOCIATED PROTEINS

Host lipids are well known to be essential components in the viral life cycle, including the assembly, budding, and replication of various viruses (Chen et al., 2005; Giese et al., 2006; Mannova et al., 2006; Oomens et al., 2006). In the case of HCV, several types of lipids are required for the HCV life cycle. Saturated and monounsaturated fatty acids, but not polyunsaturated fatty acids, enhance HCV RNA replication (Kapadia and Chisari, 2005), suggesting that lipid biogenesis is involved in HCV replication. HCV particles bind to lipoprotein receptors for entry (Agnello et al., 1999; Scarselli et al., 2002) and are believed to exist with lipoproteins in the serum of infected patients (Thomssen et al., 1992). There is also evidence that HCV uses the VLDL assembly and secretion pathway for maturation and secretion of viral particles (Huang et al., 2007; Gastaminza et al., 2008). Cholesterol and sphingolipids are employed for virion maturation and infectivity, since depletion of cholesterol or down-regulation of sphingomyelin reduces infectivity (Aizaki et al., 2008). Accumulation of lipid components in the liver leads to liver steatosis, and is associated with progression to liver fibrosis and hepatocellular carcinoma, as described above.

Hepatitis C virus replication is suppressed by an inhibitor of geranylgeranyl transferase I, but not by that of farnesyl transferase (Ye et al., 2003). Geranylgeranylate is known as an intermediate found in the mevalonate pathway and is covalently bound to various cellular proteins that are associated with plasma or the intracellular membrane (Horton et al., 2002). Immunoprecipitation analysis revealed that NS5A interacts with FBL2 (Wang et al., 2005a). The F-box motif is located in the N-terminus of FBL2, followed by 11 leucine-rich repeats (Ilyin et al., 1999) and the CAAX motif, which is thought to be modified by geranylgeranylation (Wang et al., 2005a). The F-box motif is generally essential for the formation of the ubiquitin ligase complex (Ilyin et al., 1999), suggesting that FBL2 regulates the ubiquitination of host or viral proteins through the interaction with NS5A. Another possibility is that FBL2 retains the viral replication complex by interacting with NS5A (**Figure 3**, step 3).

Screening of a genome-wide siRNA library revealed phosphatidylinositol 4-kinase III alpha (PI4KA) and COPI vesicle coat complex as a human gene associated with HCV replication (Bigger et al., 2004; Borawski et al., 2009; Li et al., 2009; Tai et al., 2009; Trotard et al., 2009; Vaillancourt et al., 2009; Reiss et al., 2011). Phosphatidylinositol 4-phosphate, which is associated with oxysterol binding protein (OSBP) and CERT (Peretti et al., 2008; Banerji et al., 2010) as described below, is increased by HCV infection (Bigger et al., 2004; Hsu et al., 2010; Reiss et al., 2011; Tai and Salloum, 2011). PI4KA is co-localized with NS5A and double stranded RNA in the replication platform composed of detergent-resistant lipid components, known as a membranous web, and is critical for HCV replication at posttranslational stages in the membranous web (Berger et al., 2009). NS5A can interact with PI4KA (Berger et al., 2011; Lim and Hwang, 2011; Reiss et al., 2011) and recruit PI4KA to the membranous web (Berger et al., 2009; Tai et al., 2009; Reiss et al., 2011; Tai and Salloum, 2011). Furthermore, PI4KA, but not phosphatidylinositol 4-kinase III beta, induces the membranous web structure under the non-replicative condition (Berger et al., 2011; Lim and Hwang, 2011; Reiss et al., 2011). Biosynthesis of phosphatidylinositol 4-phosphate by PI4KA that is recruited by NS5A in the membranous web may be required for HCV replication and can be an endogenous biomarker of the membranous web (**Figure 3**, step 3).

Vesicle-associated membrane protein-associated proteins were originally identified as proteins that bind to vesicle-associated membrane protein (VAMP) in the nematode *Aplysia* and were designated as VAMP-associated protein 33 kDa (later renamed VAP-A; Skehel et al., 1995). Furthermore, one homolog and its splicing variant were reported as VAP-B and -C, respectively (Nishimura et al., 1999). VAP is classified as a type II membrane protein, and is composed of three functional domains: major sperm protein (MSP), which occupies the N-terminal half region, the coiled-coil domain, and the transmembrane domain. VAP-A shares 60% identity with VAP-B, while VAP-C is the splicing variant of VAP-B that lacks the coiled-coil and transmembrane domains (Nishimura et al., 1999). GST pull-down and immunoprecipitation analyses revealed that NS5A and NS5B interact with human VAP-A/B and that the N-terminal MSP domain and the coiled-coil domain of VAP-A/B are responsible for the binding to NS5B and NS5A, respectively (Tu et al., 1999; Hamamoto et al., 2005). In addition, systematic RNAi screening revealed that 62 target host genes are involved in HCV RNA or proteins including VAP-A/B (Randall et al., 2007). Several reports suggest that HCV replication takes place on the membranous web (Shi et al., 2003; Gao et al., 2004; Sakamoto et al., 2005). NS4B is predominantly associated with a lipid-raft-like detergent-resistant fraction equivalent to the membranous web, and both NS5A and NS5B were co-localized in a similar fraction in the presence of NS4B (Sakamoto et al., 2005). VAP-A was also localized in a detergent-resistant fraction, suggesting that it plays an important role in HCV replication because the dominant negative mutant of VAP-A suppressed the replication of HCV RNA (Gao et al., 2004). VAP-B forms a homodimer and heterodimer with VAP-A, and knockdown of VAP-A or VAP-B led to substantial suppression of HCV replication (Hamamoto et al., 2005). These findings suggest that VAP-A and -B positively regulate HCV replication by binding to NS5A/B.

The physiological function of VAPs was reported to be trafficking of ceramide and cholesterol between ER and the Golgi apparatus. Several VAP-interacting proteins share the FFAT motif (two phenylalanines in an acidic tract), which has the consensus amino acid sequence EFFDAxE, as determined by a comparison among oxysterol binding proteins, OSBP-related proteins (ORPs; Loewen et al., 2003), and the ceramide transport protein CERT (Hanada et al., 2003; Kawano et al., 2006), contributing to the regulation of lipid metabolism. OSBP binds and transports cholesterol or hydroxycholesterol from ER to the Golgi (Ridgway et al., 1992; Wang et al., 2005b), while CERT binds and transports ceramide from ER to the Golgi, where the ceramide is converted to sphingolipids (Kumagai et al., 2005). Altering the sphingomyelin/ceramide ratio of the plasma membrane can effect HCV entry via the cell surface expression of CD81 (Voisset et al., 2008). OSBP mediates HCV secretion while binding to NS5A and VAP-A (Amako et al., 2009). Inhibition of CERT function effectively suppressed HCV release regardless of RNA replication (Aizaki et al., 2008). Phosphorylation of CERT and OSBP by protein kinase D negatively regulates VAPs binding to CERT and OSBP resulting in an effect on HCV infection (Amako et al., 2011). HCV NS5A may allow VAP-A/B to provide ceramide and cholesterol to replication complexes for upregulation of virus propagation (**Figure 3**, step 3).

The VAP-B-splicing variant VAP-C interacts with NS5B via the short form of the MSP domain and then suppresses the HCV replication by disrupting binding of other VAPs to NS5B (Kukihara et al., 2009). Expression of VAP-C is observed in various tissues except for the liver, suggesting that tissue distribution of VAP-C determines the tropism of HCV infection (Kukihara et al., 2009). These findings suggest that VAP-C negatively regulates HCV replication by inhibiting the interaction between VAP-A/B and NS5B. Furthermore, expression of VAP-C was negligible in B cells prepared from chronic hepatitis C patients, in whom B cells included HCV particles (Ito et al., 2010), and expression of the full HCV genome in B cells induced B-cell lymphoma in a conditional transgenic mouse (Kasama et al., 2010), suggesting that HCV infection increases the chance of developing B-cell lymphomas via dysregulation of lipid metabolism.

CONCLUSION

This review summarizes several recently reported viral and host factors that exploit lipid components to support HCV infection. The mechanism by which HCV proteins cooperate with host factors to exploit lipid components and to regulate lipid metabolism in the infection has not been elucidated completely. The aim of identifying host factors is effective and stable therapy; targeting the host factors might be done to prevent the emergence of resistant viruses. Cyclosporin analogs will be used clinically in the near future. Wide screening and proteomics analyses have revealed novel host factors that are required for HCV replications over the past decade. The mechanism by which HCV infection induces formation of membranous web in infected cells has been unknown yet, although NS4B is involved in formation of membranous web (Egger et al., 2002; Gosert et al., 2005; Ferraris et al., 2010). We also found several host proteins to be NS4B-associating host factors by proteomics analysis based on the TargetMine program (Tripathi et al., 2010). Further study will be required to identify the prominent factors essential for lipid metabolism that are associated with each step in the HCV life cycle and to develop effective and stable therapies for hepatitis C.

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Immunity
Article

The Transcription Factor Jdp2 Controls Bone Homeostasis and Antibacterial Immunity by Regulating Osteoclast and Neutrophil Differentiation

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SUMMARY

Jdp2 is an AP-1 family transcription factor that regulates the epigenetic status of histones. Previous in vitro studies revealed that Jdp2 is involved in osteoclastogenesis. However, the roles of Jdp2 in vivo and its pleiotropic functions are largely unknown. Here we generated *Jdp2*$^{-/-}$ mice and discovered its crucial roles not only in bone metabolism but also in differentiation of neutrophils. *Jdp2*$^{-/-}$ mice exhibited osteopetrosis resulting from impaired osteoclastogenesis. *Jdp2*$^{-/-}$ neutrophils were morphologically normal but had impaired surface expression of Ly6G, bactericidal function, and apoptosis. We also found that ATF3 was an inhibitor of neutrophil differentiation and that Jdp2 directly suppresses its expression via inhibition of histone acetylation. Strikingly, *Jdp2*$^{-/-}$ mice were highly susceptible to *Staphylococcus aureus* and *Candida albicans* infection. Thus, Jdp2 plays pivotal roles in in vivo bone homeostasis and host defense by regulating osteoclast and neutrophil differentiation.

INTRODUCTION

Jun dimerization protein 2 (Jdp2) is a member of the AP-1 family and interacts with other AP-1 components, such as c-Jun, JunB, JunD, and ATF2 (Aronheim et al., 1997). Jdp2 can inhibit the activation of its binding partners, suggesting that it is a transcriptional repressor (Jin et al., 2001). Furthermore, Jdp2 suppresses histone acetyltransferase activity and acetylation of reconstituted nucleosomes, thereby regulating the epigenetic status of histones (Jin et al., 2006). Extensive studies have revealed that Jdp2 plays roles in various cellular responses, such as UV-induced apoptosis and osteoclastogenesis (Huang et al., 2010).

Osteoclasts are multinucleated cells that degrade bone (Karsenty and Wagner, 2002). Bone-forming osteoblasts express macrophage colony-stimulating factor (M-CSF) and RANK ligand (RANKL). When these cytokines stimulate their receptors, c-fms and RANK, respectively, transcription factors such as c-Fos, NF-κB, and NFATc1 (Takayanagi, 2007) are activated in osteoclast precursors and osteoclastogenesis is induced by stimulation of osteoclastogenic genes, such as tartrate-resistant acid phosphatase (TRAP) and cathepsin K (CTSK). Jdp2 was previously implicated in positive regulation of osteoclastogenesis via activation of the TRAP and CTSK promoters (Kawaida et al., 2003). Recent findings indicate that the Jdp2 locus is hypomethylated and that its transcript is upregulated in common myeloid precursors and granulocyte-macrophage progenitors relative to lymphoid lineages (Ji et al., 2010), suggesting that Jdp2 may also contribute to the differentiation of myeloid cells, such as neutrophils.

Neutrophils are critical for bacterial clearance. One of the most impressive morphological features of mature neutrophils is cytosolic granules, and the mRNA expressions of granule content genes are significantly higher in immature neutrophils than in mature neutrophils (Borregaard and Cowland, 1997; Borregaard et al., 2007). There are three different granule subtypes, i.e., primary, secondary, and tertiary, and the granule proteins play pivotal roles in bacterial killing. The other bactericidal agents derived from neutrophils are reactive oxygen species (ROS), such as superoxide (Forman and Thomas, 1986). Recently, a novel mechanism of bacterial and fungal killing mediated by chromatin structures was elucidated, termed the neutrophil extracellular trap (NET) (Brinkmann et al., 2004). This extracellular structure is released through a cell death requiring ROS production (Nishinaka et al., 2011) and chromatin decondensation (Li et al., 2010). Collectively, these findings demonstrate that neutrophils exert bactericidal activity through several complex machineries.

Generally, neutrophil subtypes can be distinguished by their surface markers CD11b and Ly6G. CD11b^{+}Ly6G^{lo} cells are immature neutrophils with a round nucleus, such as myelocytes, whereas CD11b^{+}Ly6G^{hi} cells are band-segmented mature neutrophils (Hestdal et al., 1991). By using such morphological and molecular cues, several studies have shown that various cytokines and transcription factors are critical for proper

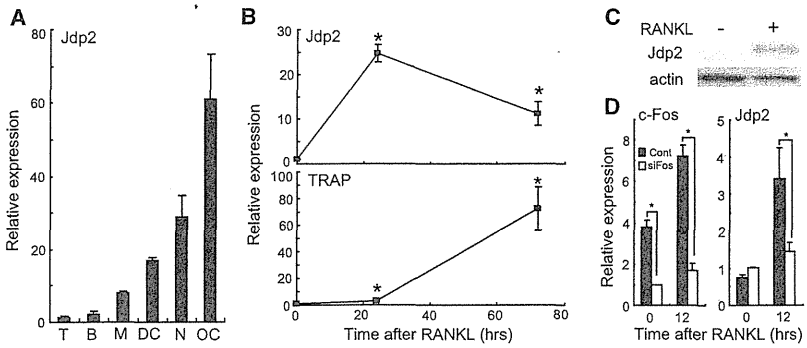

Figure 1. Jdp2 Expression

(A) qPCR analysis of Jdp2 in splenic T cells (T), B cells (B), DCs (DC), neutrophils (N), and primary bone marrow osteoclasts (OC) (n = 3).

(B) qPCR analysis of Jdp2 in MDMs in response to RANKL stimulation. *p < 0.05 versus 0 hr (n = 3).

(C) MDMs were stimulated with 50 ng/ml RANKL for 30 hr. Jdp2 levels were analyzed by protein immunoblotting.

(D) MDMs were transfected with control siRNA (Cont) or c-Fos-specific siRNA (siFos) and stimulated with 50 ng/ml RANKL for 12 hr. c-Fos and Jdp2 levels were measured by qPCR (n = 3).

*p < 0.05 versus control siRNA. Error bars, SE.

development of neutrophils. For example, granulocyte colony-stimulating factor (G-CSF) plays a pivotal role in proliferation of neutrophil precursors via activation of STAT3 (Lieschke et al., 1994). The CCAAT/enhancer binding protein (C/EBP) family is also critically involved in neutrophil differentiation. In particular, C/EBPα is considered a master regulator of neutrophils because C/EBPα-deficient mice lack neutrophils (Zhang et al., 1998). C/EBPε is involved in proper neutrophil differentiation, because neutrophils from C/EBPε-deficient mice have abnormal respiratory burst activity and lack secondary and tertiary granules (Yamanaka et al., 1997). Recent reports have also implicated the transcription factors Gfi-1 (Hock et al., 2003) and Ikaros (Dumortier et al., 2003) in proper differentiation of neutrophils. Overall, these findings suggest that neutrophil differentiation is orchestrated by interplay among several transcription factors.

Despite its importance in in vitro osteoclastogenesis and several implications for its activity in myeloid lineage cells, the roles of Jdp2 in vivo and its pleiotropic functions are completely unknown. Here, we generated *Jdp2*$^{-/-}$ mice and discovered critical roles of Jdp2 not only in bone homeostasis but also in proper differentiation of neutrophils.

RESULTS

Jdp2$^{-/-}$ Mice Are Osteopetrotic because of Impaired Osteoclastogenesis

First, we examined Jdp2 expression in mature myeloid cells, such as macrophages, dendritic cells (DCs), neutrophils, and osteoclasts. Jdp2 expression was substantially higher in these cells than in lymphoid cells, such as T and B cells (Figure 1A). Because Jdp2 expression was highest in osteoclasts (Figure 1A), we focused on the regulation of Jdp2 expression in response to RANKL. Jdp2 expression was significantly increased in M-CSF-derived macrophages (MDMs) after RANKL stimulation (Figures 1B and 1C) but not after LPS stimulation (Figure S1 available online). This transcriptional induction was dependent on c-Fos (based on siRNA knockdown), which is recognized as a pivotal transcription factor for osteoclastogenesis (Figure 1D). The existence of this c-Fos-Jdp2 axis prompted us to explore the role of Jdp2 in RANKL-induced osteoclastogenesis.

To evaluate osteoclastogenesis in vitro, *Jdp2*$^{-/-}$ mice were generated (Figures S2A–S2C). Surprisingly, in vitro RANKL-induced osteoclastogenesis and resorption pit formation were completely abrogated in *Jdp2*$^{-/-}$ cells (Figure 2A). We also evaluated the characteristics of splenic macrophages and found that the populations were similar between wild-type and *Jdp2*$^{-/-}$ cells (Figures S2D, S2E, and S2G). In addition, *Jdp2*$^{-/-}$ MDMs exhibited normal proliferation (Figure S2F) and RANK and c-fms expression (Figure S2H).

As previously reported (Kawaida et al., 2003), induction of osteoclast-associated genes, including TRAP and CTSK, was abrogated in *Jdp2*$^{-/-}$ cells, whereas c-Fos induction was comparable between wild-type and *Jdp2*$^{-/-}$ cells (Figure 2B). We also found that induction of NFATc1 mRNA and DNA binding of NFATc1 to its promoter was partially suppressed in *Jdp2*$^{-/-}$ cells in response to RANKL (Figures S2I and S2J). In addition, Jdp2 had no effect on NFATc1 binding to its promoter region (Figure S2K). Furthermore, DNA binding of NF-κB p65 was normal (Figure S2L). The expression levels of Blimp1, a positive regulator of osteoclastogenesis (Nishikawa et al., 2010), and Blimp1 target genes, such as *Irf8* and *Bcl6*, were comparable (Figure S2M). RANKL-induced calcium oscillation was normal in *Jdp2*$^{-/-}$ cells (Figure S2N). Because TREM2 is required for osteoclast multinucleation (Humphrey et al., 2006), we examined the TREM2 expression levels in wild-type and *Jdp2*$^{-/-}$ MDMs but found no difference (Figure S2O). When TREM2 was stimulated by antibody, wild-type MDMs formed increased numbers of osteoclasts. In contrast, TREM2 stimulation had no effect on osteoclastogenesis of *Jdp2*$^{-/-}$ MDMs (Figures S2P and S2Q). Finally, retrovirus reconstitution of Jdp2 in *Jdp2*$^{-/-}$ MDMs rescued RANKL-induced osteoclastogenesis (Figures S2R and S2S). Together, these results indicate that the c-Fos-Jdp2 axis is critical for controlling osteoclastogenesis via proper induction of NFATc1 and osteoclastogenic genes, such as *TRAP* and *CTSK*.

These findings prompted us to explore the role of Jdp2 in in vivo bone homeostasis. No apparent abnormalities were observed in *Jdp2*$^{-/-}$ mice, although they did exhibit slightly shortened femurs (Figure 2D). Radiographic analysis of the femurs showed that *Jdp2*$^{-/-}$ mice had osteopetrosis accompanied by marked increases in trabecular bone volume and number, compared with wild-type mice (Figures 2C, 2F, and 2G). These findings were further supported by increased bone mineral density (BMD) in the full-length femurs of *Jdp2*$^{-/-}$ mice (Figure 2E). Sections of proximal tibias from *Jdp2*$^{-/-}$ mice also showed increased trabecular bone volume and number (Figures 2H and 2I). Histomorphometric analysis revealed

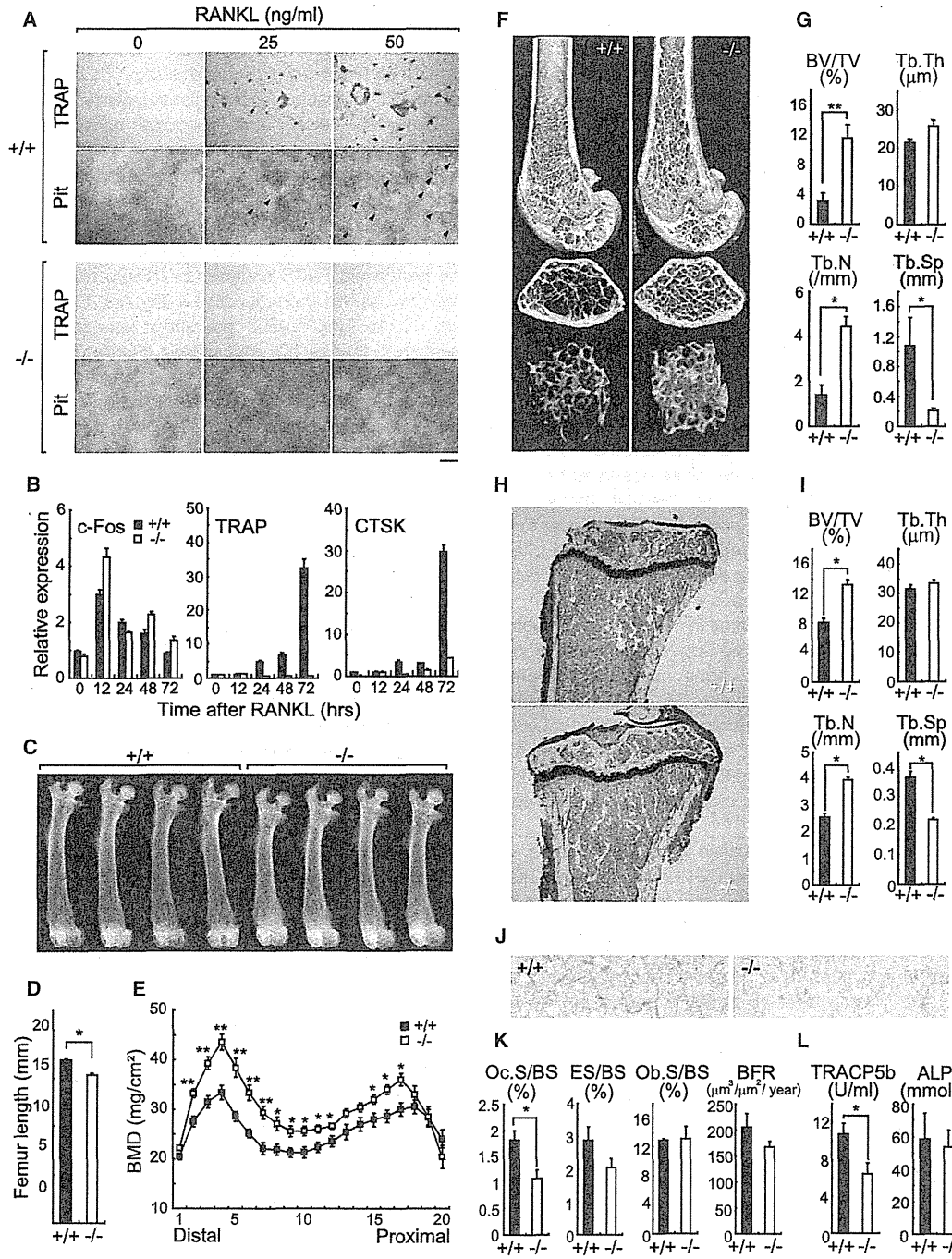

Figure 2. Impaired Osteoclastogenesis in *Jdp2*$^{-/-}$ Mice

(A) MDMs from wild-type and *Jdp2*$^{-/-}$ mice were cultured with the indicated concentrations of RANKL. Representative TRAP staining and resorption pits (arrowheads) are shown. Scale bar represents 200 μm.

(B) qPCR analysis of c-Fos, TRAP, and CTSK in wild-type and *Jdp2*$^{-/-}$ MDMs stimulated with 50 ng/ml RANKL (n = 3).

(C) Soft X-ray images of femurs.

(D) Femur lengths.

(E) BMDs of 20 longitudinal femur divisions.

(F) Representative μCT images of distal femurs (top, longitudinal view; middle, axial view of metaphyseal region; bottom, 3D view of metaphyseal region).

(G) Bone morphometric analysis of distal femurs by μCT.

a significant reduction in the osteoclast surface/bone surface ratio in *Jdp2*$^{-/-}$ mice, whereas the osteoblast surface/bone surface ratio and bone formation rate were normal (Figures 2J and 2K). Consistent with the decreased osteoclastogenesis in vivo, the serum bone resorption marker TRACP5b was lower in *Jdp2*$^{-/-}$ mice (Figure 2L). Furthermore, wild-type mice engrafted with bone marrow cells from *Jdp2*$^{-/-}$ mice had an increased bone volume phenotype (Figures S2T and S2U). Collectively, these results indicate that Jdp2 is critical for controlling osteoclastogenesis both in vitro and in vivo.

Neutrophils in *Jdp2*$^{-/-}$ Mice Are Morphologically Normal but Show Impaired Ly6G Expression

A recent report suggested that Jdp2 may be involved in the choice between lymphoid or myeloid differentiation (Ji et al., 2010). Therefore, we focused on the populations of lymphoid and myeloid cells (Figure S3A). No differences in the expressions of cell surface phenotype markers and numbers and ratios of T cells, B cells, and DCs in the spleen were observed between wild-type and *Jdp2*$^{-/-}$ mice (Figure S3A). We also checked the cytokine production (Figures S3B and S3C), bactericidal function (Figure S3D), superoxide production (Figure S3E), and phagocytosis activity (Figure S3F) in *Jdp2*$^{-/-}$ MDMs and conventional DCs (cDCs), and all phenotypes were normal.

Because Jdp2 was highly expressed in mature splenic neutrophils (Figure 1A), we compared the Jdp2 mRNA and protein expression between splenic and bone marrow neutrophils. Jdp2 expression in bone marrow mature CD11b^{+}Ly6G^{hi} neutrophils was lower than that in splenic mature CD11b^{+}Ly6G^{hi} neutrophils, but higher than that in bone marrow immature CD11b^{+}Ly6G^{lo} neutrophils (Figures S3G and S3H). These findings suggest that Jdp2 gradually increases during neutrophil differentiation and maturation.

To check the maturity of neutrophils from *Jdp2*$^{-/-}$ mice, we performed FACS analyses by using CD11b and Ly6G markers (Figure 3A). In *Jdp2*$^{-/-}$ bone marrow cells, the proportion of the CD11b^{+}Ly6G^{hi} population was shifted toward the CD11b^{+}Ly6G^{lo} population (Figure 3A). This low level of Ly6G indicated accumulation of immature cells. However, contrary to our expectation, *Jdp2*$^{-/-}$ neutrophils displayed a normal segmented nuclear morphology (Figure 3A). Because the CD11b^{+} population includes a Ly6C^{hi}Ly6G^{lo} inflammatory monocyte population (Colonna et al., 2004; Lagasse and Weissman, 1996) and a Ly6C^{lo}Ly6G^{+} neutrophil population, CD11b^{+} cells were further gated on CD11b^{+}Ly6C^{lo}Ly6G^{+} neutrophils and CD11b^{+}Ly6C^{hi}Ly6G^{lo} inflammatory monocytes to exclude monocytes from the *Jdp2*$^{-/-}$ CD11b^{+}Ly6G^{+} population (Figure 3B). Among CD11b^{+}Ly6C^{lo}Ly6G^{+} neutrophil populations, the CD11b^{+}Ly6C^{lo}Ly6G^{hi} population was shifted to the CD11b^{+}Ly6C^{lo}Ly6G^{lo} population in *Jdp2*$^{-/-}$ mice (Figure 3B). In contrast, CD11b^{+}Ly6C^{hi}Ly6G^{lo} inflammatory monocyte populations were comparable between wild-type and *Jdp2*$^{-/-}$ cells (Figure 3B). We also confirmed that both wild-type and *Jdp2*$^{-/-}$ CD11b^{+}Ly6C^{lo}Ly6G^{+} neutrophil populations had similar segmented nuclei (Figure 3B).

To further assess the abnormal bone marrow CD11b^{+}Ly6C^{lo}Ly6G^{+} neutrophil population in *Jdp2*$^{-/-}$ mice, we analyzed the cellular microstructure by transmission electron microscopy (TEM) (Figure 3C). However, the intracellular morphology of *Jdp2*$^{-/-}$ cells seemed normal (Figure 3C). To determine whether the abnormal neutrophils accumulated only in the bone marrow, we performed FACS analyses of thioglycollate-elicited peritoneal neutrophils and splenocytes (Figures 3D and 3E). An atypical CD11b^{+}Ly6C^{lo}Ly6G^{lo} population was observed in *Jdp2*$^{-/-}$ peritoneal (Figure 3D) and splenic (Figure 3E) neutrophils. To determine whether the defect in neutrophils in *Jdp2*$^{-/-}$ mice was bone marrow derived and cell intrinsic, we engrafted irradiated wild-type mice with *Jdp2*$^{-/-}$ or wild-type bone marrow. After reconstitution, we observed the same phenotype of neutrophils in the wild-type mice with *Jdp2*$^{-/-}$ bone marrow as in the *Jdp2*$^{-/-}$ mice (Figures S3O–S3Q). Together, these findings suggest that *Jdp2*$^{-/-}$ neutrophils are morphologically normal but have diminished Ly6G expression and that this abnormality arises in a cell-intrinsic manner.

Impaired Apoptosis and Bactericidal Function in *Jdp2*$^{-/-}$ Neutrophils

Intriguingly, we observed slight increases in CD11b^{+}Ly6C^{lo}Ly6G^{+} neutrophil numbers (~20%) in *Jdp2*$^{-/-}$ bone marrow and peripheral populations (Figures 3A, 3B, and 3E). Given this observation, we examined the spontaneous apoptosis of *Jdp2*$^{-/-}$ peritoneal neutrophils (Figure 3F). To our surprise, *Jdp2*$^{-/-}$ neutrophils showed impaired apoptosis compared with wild-type neutrophils (Figure 3F). Microarray and quantitative PCR (qPCR) analyses of neutrophils revealed that Bcl-2 expression was significantly increased in *Jdp2*$^{-/-}$ neutrophils, whereas Jdp2 deficiency had no effect on the diverse array of other Bcl-2-associated genes (Figures 3G–3I). Next, several assays were used to examine *Jdp2*$^{-/-}$ peritoneal neutrophil function. First, the capacity of *Jdp2*$^{-/-}$ mice to recruit neutrophils into the peritoneal cavity after thioglycollate injection was determined, with no difference in cell numbers found between wild-type and *Jdp2*$^{-/-}$ mice (Figure S3I). Second, we checked the cytokine production by *Jdp2*$^{-/-}$ neutrophils and found that Jdp2 deficiency did not alter cytokine production in response to TLR ligands (Figure S3J). Third, we analyzed the function of Jdp2 in NET formation. Intriguingly, we observed a 50% reduction in NET formation in neutrophils from *Jdp2*$^{-/-}$ mice in response to *Staphylococcus aureus* and *Candida albicans* infection (Figures 3J–3M). Fourth, because ROS, such as superoxide, are required for NET formation, we quantified superoxide production by *Jdp2*$^{-/-}$ neutrophils in

(H) Representative proximal tibias.
(I) Bone morphometric analysis of proximal tibias.
(J) TRAP staining of metaphyseal portions of tibias.
(K) Bone histomorphometric analysis of metaphyseal portions of tibias.
(L) Serum levels of TRACP5b and alkaline phosphatase (ALP).
Abbreviations: BV/TV, bone volume per tissue volume; Tb.Th, trabecular bone thickness; Tb.N, trabecular bone number; Tb.Sp, trabecular bone spacing; Oc.S/BS, osteoclast surface per bone surface; ES/BS, eroded surface per bone surface; Ob.S/BS, osteoblast surface per bone surface; BFR, bone formation rate. Error bars, SE. *$p < 0.05$; **$p < 0.01$ ($n = 4$).

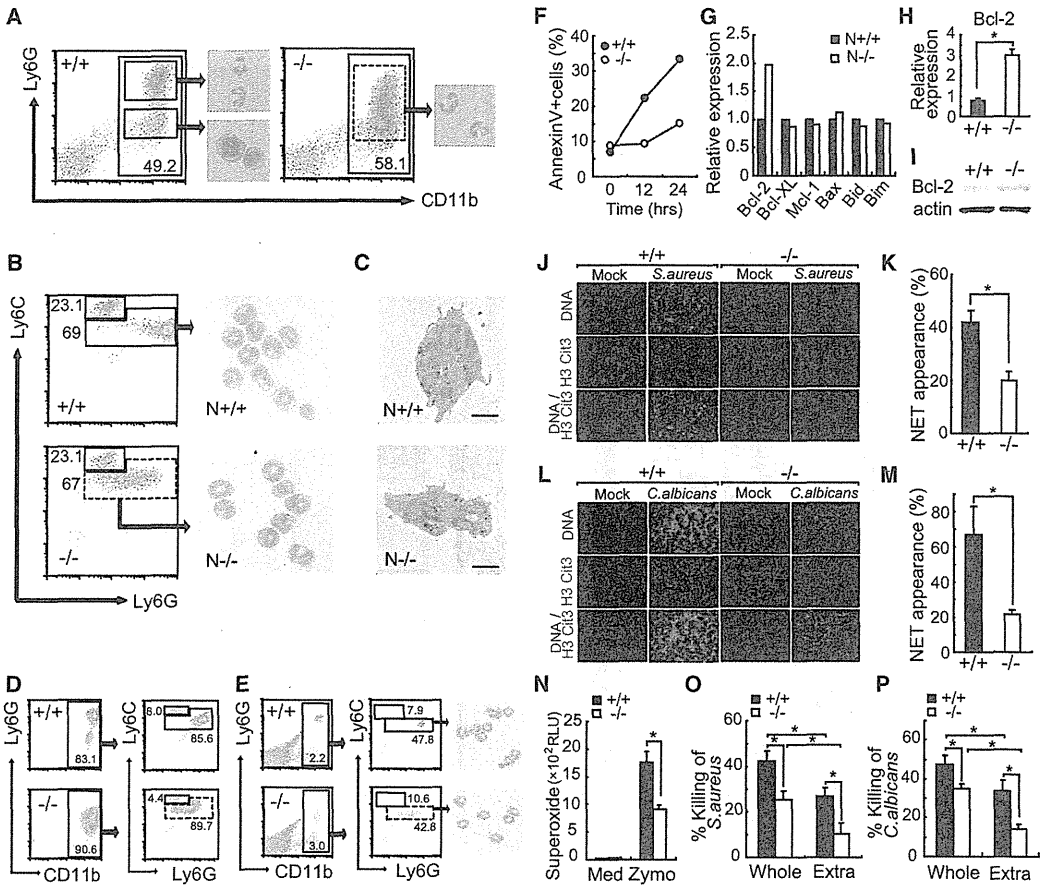

Figure 3. Abnormal Phenotype of *Jdp2*$^{-/-}$ Neutrophils

(A) FACS analysis of wild-type and *Jdp2*$^{-/-}$ bone marrow cells via Ly6G and CD11b markers. Gated cells were sorted and stained with May-Grunwald-Giemsa.

(B) CD11b^{+} populations in (A) were further analyzed with Ly6C marker. CD11b^{+}Ly6C^{lo}Ly6G^{+} neutrophils (N+/+ and N−/−) were sorted and stained as in (A).

(C) N+/+ and N−/− cells were fixed, stained with diaminobenzidine, and analyzed by TEM. Scale bars represent 2 μm.

(D and E) Peritoneal neutrophils (D) and splenocytes (E) were analyzed as in (B).

(F) Peritoneal neutrophils were cultured in vitro and analyzed for the percentage of annexin V-positive cells by FACS (n = 3 independent experiments).

(G) mRNA levels of apoptosis-regulating genes in bone marrow CD11b^{+}Ly6C^{lo}Ly6G^{+} neutrophils (N+/+ and N−/−) analyzed by a microarray.

(H and I) Bcl2 mRNA (H) and protein (I) expression levels in wild-type and *Jdp2*$^{-/-}$ peritoneal neutrophils were analyzed by qPCR and protein immunoblotting, respectively (n = 3).

(J) Peritoneal neutrophils were infected by *S. aureus* for 2 hr (MOI = 50) and stained by Hoechst and anti-histone H3 Cit3 Ab. DNA-histone H3 Cit3 Ab double-positive structures were defined as NETs.

(K) 50 microscopic fields (40×) in wells containing *S. aureus*-infected neutrophils, shown in (J), were checked and the rate of NET appearance was calculated (n = 4 observations).

(L) Peritoneal neutrophils were infected by *C. albicans* for 2 hr (MOI = 50) and stained as in (J).

(M) *C. albicans*-induced NET formation in (L) was measured as in (K).

(N) Peritoneal neutrophils were stimulated with 100 μg/ml Zymosan for 15 min and supernatant superoxide levels were measured.

(O) *S. aureus* killing by peritoneal neutrophils. Phagocytosis was inhibited by cytochalasin D and bacterial killing was measured (Extra) (n = 6).

(P) *C. albicans* killing by peritoneal neutrophils was determined as in (O).

Error bars, SE. *p < 0.05.

response to Zymosan and Curdlan. We observed 50% reductions in superoxide production in *Jdp2*$^{-/-}$ neutrophils (Figures 3N and S3L). We also checked the expression of Dectin-1, a Curdlan receptor, and observed similar expression levels between wild-type and *Jdp2*$^{-/-}$ neutrophils (Figure S3K). To clarify the mechanisms of decreased superoxide production in Jdp2 deficiency, we checked the expression levels of NADPH oxidase subunits and found that NCF1 expression was lower in *Jdp2*$^{-/-}$ neutrophils than in wild-type cells (Figure S3M). Therefore, we infected *Jdp2*$^{-/-}$ neutrophils with a retrovirus encoding NCF1 and measured the superoxide production. However, the rescue of *Jdp2*$^{-/-}$ neutrophils by NCF1 was less efficient than that by Jdp2 (Figure S3N). Thus, increased NCF1 can partially rescue the impaired superoxide production in *Jdp2*$^{-/-}$ neutrophils. Finally, to determine whether the functional defects of *Jdp2*$^{-/-}$ neutrophils were associated with bacterial killing

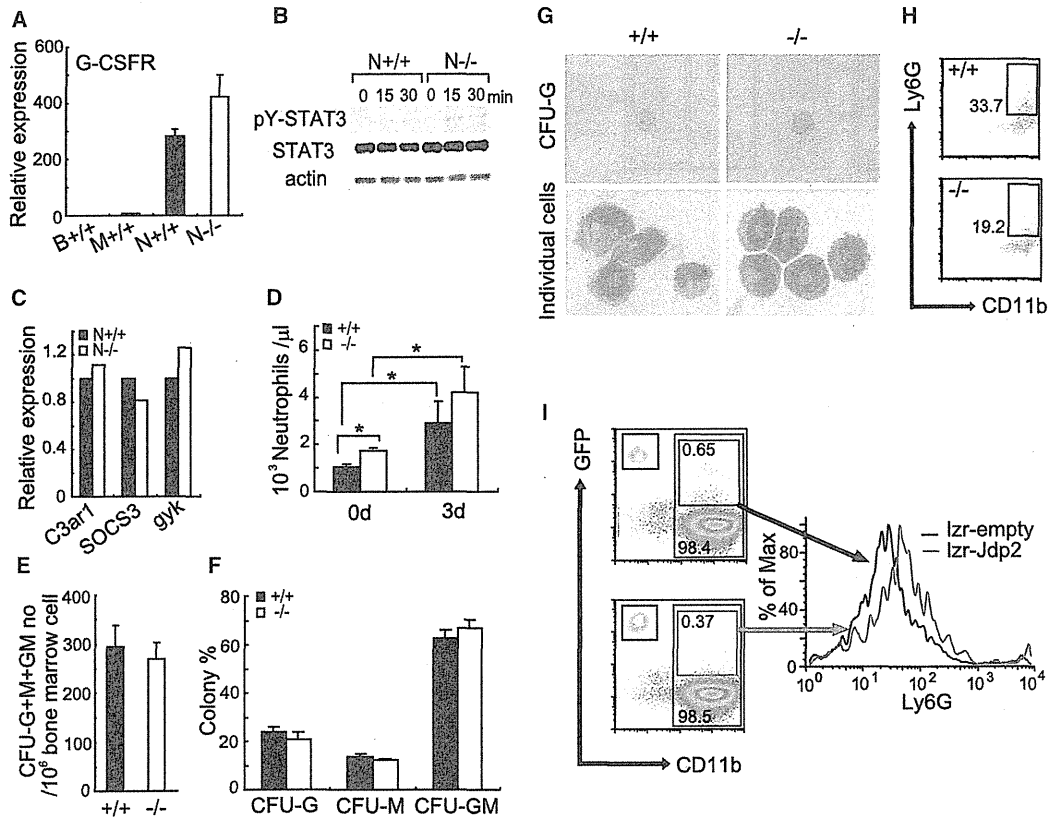

Figure 4. G-CSF Response Kinetics of *Jdp2*$^{-/-}$ Cells

(A) G-CSFR mRNA levels in wild-type and *Jdp2*$^{-/-}$ bone marrow CD11b^{+}Ly6C^{lo}Ly6G^{+} neutrophils (N+/+ and N−/−) measured by qPCR. Error bars, SE (n = 3).

(B) Cells in (A) were stimulated with 100 ng/ml G-CSF. STAT3 and pY-STAT3 levels were detected by immunoblotting.

(C) mRNA levels of STAT3 target genes in CD11b^{+}Ly6C^{lo}Ly6G^{+} neutrophils (N+/+ and N−/−) analyzed by a microarray.

(D) G-CSF (1 μg) was subcutaneously injected into wild-type and *Jdp2*$^{-/-}$ mice from days 0 to 3. At 6 hr after the last injection, blood was collected and CD11b^{+}Ly6G^{+} neutrophils were counted. Error bars, SE (n = 3). *p < 0.05.

(E and F) Bone marrow cells were cultured for 7 days in MethoCult. Total numbers of CFU-G, CFU-M, and CFU-GM colonies (E) and their rates (F) were determined. Error bars, SE (n = 6).

(G) Bone marrow cells were cultured for 7 days in MethoCult with 50 ng/ml G-CSF. Representative images of CFU-G and diaminobenzidine plus May-Grunwald-Giemsa-stained individual cells are indicated.

(H) CFU-G in (G) were collected and analyzed by FACS with CD11b and Ly6G markers.

(I) *Jdp2*$^{-/-}$ bone marrow cells were infected with a retrovirus encoding Jdp2 and GFP (Izr-Jdp2) or GFP alone (Izr-empty) with G-CSF for 9 days. CD11b^{+}GFP^{+} cells were gated and Ly6G expression levels were quantified by FACS. Gated cells were also sorted and stained by May-Grunwald-Giemsa (upper left insets in the scatter plots).

deficits, we performed in vitro killing assays with *S. aureus* and *C. albicans* (Figures 3O and 3P). Phagocytosis-dependent intracellular killing was inhibited by pretreating neutrophils with cytochalasin D. We observed that component killing mainly occurred in the extracellular space (Figures 3O and 3P) and that whole and extracellular bacterial killing by neutrophils from *Jdp2*$^{-/-}$ mice was significantly decreased compared with wild-type mice (Figures 3O and 3P). Together, these results clearly indicate that the bactericidal function is impaired in *Jdp2*$^{-/-}$ neutrophils.

Abnormal Differentiation of *Jdp2*$^{-/-}$ Neutrophils In Vitro Is Corrected by Re-expression of Jdp2

To investigate whether G-CSF signaling is altered by Jdp2 deficiency, we examined the expression levels of G-CSF receptor and STAT3. We found that G-CSF receptor (G-CSFR) expression was comparable between wild-type and *Jdp2*$^{-/-}$ neutrophils (Figure 4A), as was the expression of both STAT3 and phosphorylated STAT3 (Figure 4B). Microarray data confirmed the normal expression levels of G-CSF target genes (Figure 4C). We also counted the blood neutrophil numbers after intraperitoneal G-CSF injection and found comparable increasing rates of neutrophil numbers between wild-type and *Jdp2*$^{-/-}$ mice (Figure 4D). Thus, loss of Jdp2 does not influence G-CSF signaling.

Subsequently, we examined whether the altered differentiation of neutrophils in *Jdp2*$^{-/-}$ mice was a late-phase abnormality. *Jdp2*$^{-/-}$ bone marrow cells gave rise to the same numbers of granulocyte colony-forming units (CFU-G), granulocyte-macrophage colony-forming units (CFU-GM), and macrophage

colony-forming units (CFU-M) as did wild-type cells, with similar rates of formation (Figures 4E and 4F). We also added G-CSF to stem cell medium and cultured bone marrow cells (Figure 4G). As expected, the colony numbers (data not shown) and morphology (Figure 4G) between the two cell types were similar, but Ly6G expression was decreased in cells derived from *Jdp2*$^{-/-}$ bone marrow colonies (Figure 4H). Together, our findings imply that the abnormality in neutrophils from *Jdp2*$^{-/-}$ mice arises in the late differentiation phase and not in the initial differentiation phase.

Finally, we determined whether reintroduction of Jdp2 could rescue the terminal differentiation. We infected *Jdp2*$^{-/-}$ bone marrow cells with a retrovirus encoding Jdp2 and GFP or GFP alone and cultured the cells in medium containing G-CSF (Figure 4I). After 9 days, the cells were harvested and their Ly6G expression levels in gated GFP-positive neutrophils were quantified by FACS (Figure 4I). As expected, *Jdp2*$^{-/-}$ bone marrow-derived neutrophils infected with the Jdp2-GFP retrovirus exhibited increased Ly6G expression compared with control GFP-only cells (Figure 4I). Thus, the defect in neutrophil differentiation in *Jdp2*$^{-/-}$ mice appears to be cell autonomous and can be corrected by re-expression of Jdp2.

Primary Granule mRNA Expression Is Elevated in *Jdp2*$^{-/-}$ Neutrophils

The mRNA levels of granule genes are higher in immature neutrophils than in mature neutrophils (Martinelli et al., 2004). Therefore, we analyzed the diverse RNAs of CD11b^{+}Ly6C^{lo}Ly6G^{+} bone marrow neutrophils encoding primary, secondary, and tertiary granules by using microarray data (Figure 5A). Intriguingly, the mRNA levels for primary granule proteins, such as MPO, CTSG, and PR3, were significantly increased in *Jdp2*$^{-/-}$ neutrophils, whereas those for secondary and tertiary granule proteins were comparable to control cells (Figure 5A). The expression of other bactericidal granule proteins, such as Lipocalin2 and Cramp, was comparable (Figure S4A). We confirmed these aberrant primary granule expressions in bone marrow and peritoneal neutrophils by qPCR (Figures 5B and 5C). However, in immunoblotting analyses, the expression levels of primary granule proteins (Figure 5D) and their degranulation in response to LPS (Figure S4B) seemed comparable between wild-type and *Jdp2*$^{-/-}$ neutrophils.

To reveal the mechanism of the aberrant mRNA expression in *Jdp2*$^{-/-}$ neutrophils, we selected a set of genes whose expression levels were more abundant in *Jdp2*$^{-/-}$ neutrophils than in wild-type cells (Figure S4C) based on microarray data and examined their promoters for the presence of transcription factor binding sites. The analysis revealed that C/EBP binding sequences were highly enriched in the promoters of Jdp2-regulated genes compared with randomly selected gene promoters (Figures S4C–S4E). Further, we found that C/EBP binding sites were most enriched among 198 transcription factor binding sequences tested (Table S1). Thus, we quantified the mRNAs of the C/EBP gene family involved in myeloid differentiation. However, their expression levels were comparable (Figure 5E). C/EBPα was reported to be the master regulator of the expression of primary granule genes (Zhang et al., 1998). Therefore, we examined the DNA-binding activities of C/EBPα and C/EBPβ to their consensus oligonucleotides by using ELISA-based transcription factor kits (Figure 5F). Although the protein expression levels were again comparable (Figure 5G), C/EBPα, but not C/EBPβ, DNA binding was increased in *Jdp2*$^{-/-}$ neutrophils (Figure 5F). In addition, Jdp2 binding to the C/EBPα promoter was not detected by chromatin immunoprecipitation (ChIP) analyses (Figure 5H). When GFP-fused Jdp2 was retrovirally overexpressed in primary neutrophils, its expression was restricted to the nucleus (Figure S4F). These observations led us to examine the binding of Jdp2 to C/EBPα, and an association between C/EBPα and Jdp2 was found by immunoprecipitation (Figures 5I and 5J). From this, we examined the effect of Jdp2 on the transcriptional activity of C/EBPα (Figure 5K). For this experiment, we used a luciferase reporter plasmid driven by C/EBP transcriptional response elements. Overexpression of the C/EBPα gene only activated this promoter, whereas simultaneous expression of Jdp2 dose dependently reduced the activity of the promoter to the control level (Figure 5K). Together, these findings suggest that Jdp2 inhibits the transcriptional activity of C/EBPα by directly binding to the gene and inhibiting C/EBPα from binding to its target sequence. We also overexpressed C/EBPα in wild-type bone marrow cells and found that C/EBPα enhanced primary granule mRNAs (Figures S4G and S4H). Furthermore, when we re-expressed Jdp2 in *Jdp2*$^{-/-}$ bone marrow cells, DNA binding of C/EBPα and expression of primary granule genes were downregulated (Figures S4I and S4J). We also overexpressed C/EBPα in wild-type differentiated neutrophils (Figure S4K) and found that expression of Bcl-2 (Figure S4M) but not Ly6G (Figure S4L) was induced, leading to impaired apoptosis (Figure S4N). Together, our observations strengthen the idea that *Jdp2*$^{-/-}$ neutrophils are immature and suggest that increased primary granule and Bcl-2 mRNA expressions are attributable to increased C/EBPα activation.

ATF3 Is a Target of Jdp2 and Regulates Ly6G Expression

Among AP-1 family members, ATF3 is the closest relative of Jdp2 (Figure 6A). This information prompted us to measure ATF3 expression in neutrophils. ATF3 expression in bone marrow and peritoneal neutrophils was significantly increased (Figure 6B). We also overexpressed Jdp2 in wild-type and *Jdp2*$^{-/-}$ neutrophils and found that Jdp2 suppressed ATF3 expression (Figures 6C and 6D). Jdp2 is known to act as an epigenetic regulator of gene expression (Jin et al., 2006). Therefore, we analyzed the genome-wide status of histone acetylation, H3K4 trimethylation, and H3K27 trimethylation in wild-type and *Jdp2*$^{-/-}$ peritoneal neutrophils by using the ChIP-sequencing (ChIP-Seq) technique (Figure 6E). First, genes were chosen based on their differences in expression in wild-type and *Jdp2*$^{-/-}$ neutrophils. However, we did not find an apparent correlation between epigenetic statuses (data not shown). Moreover, primary granule genes did not have significant peaks for acetyl-histone, H3K4me3, and H3K27me3 in either wild-type or *Jdp2*$^{-/-}$ peritoneal neutrophils (Figure S5), indicating that expression of these genes is not regulated by the epigenetic status. When we focused on the ATF3 locus, we found a dramatic increase in the acetyl-histone status of the promoter region close to the transcription start site (Figure 6E). However, the same region had comparable H3K4me3 and H3K27me3 statuses (Figure 6E). By ChIP analyses, we confirmed an increase in the acetyl-histone status at the ATF3 promoter region in *Jdp2*$^{-/-}$ peritoneal neutrophils and observed

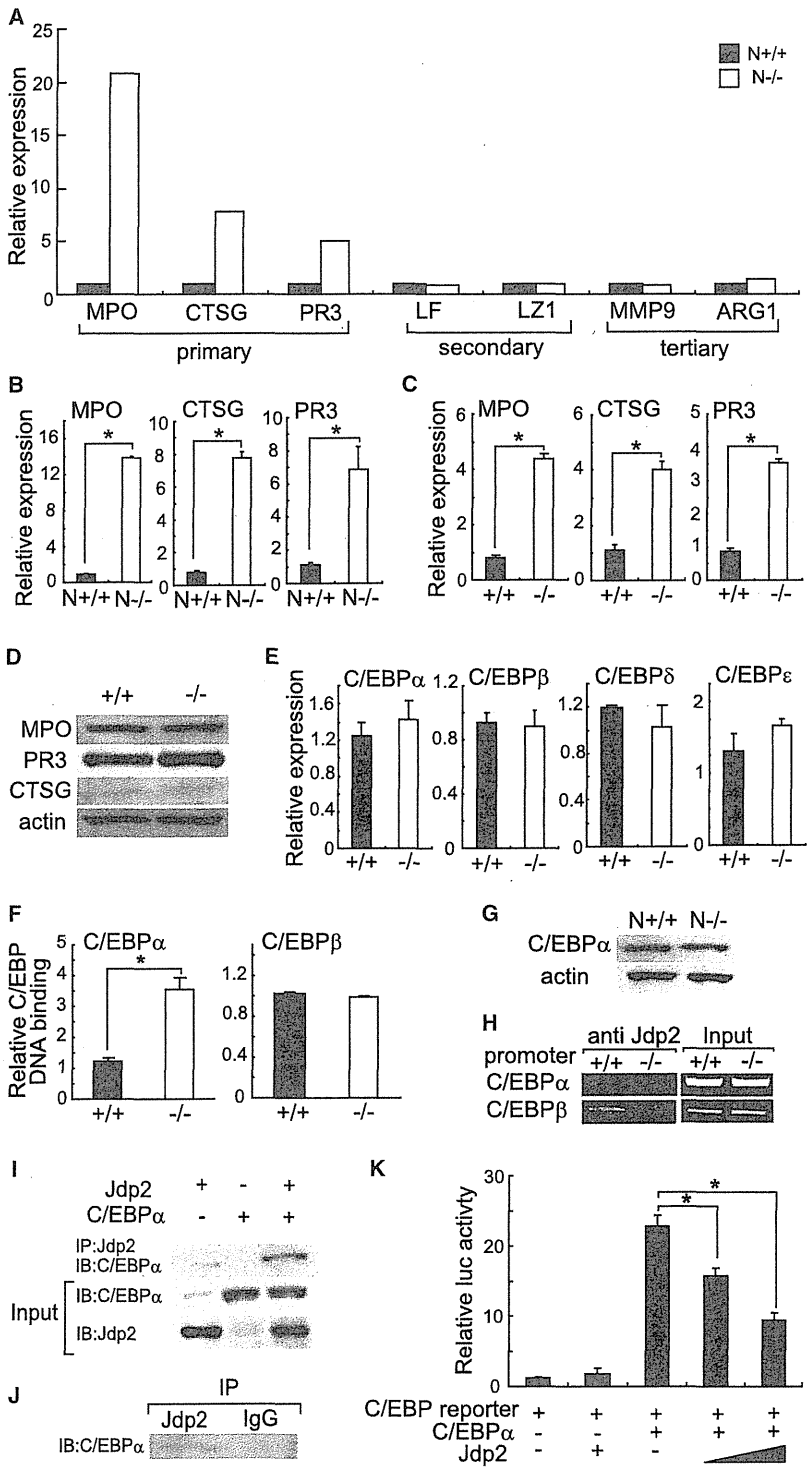

Figure 5. Aberrant mRNA Levels of Primary Granule Genes in *Jdp2*$^{-/-}$ Neutrophils

(A) Primary, secondary, and tertiary granule mRNA levels in bone marrow CD11b^{+}Ly6C^{lo}Ly6G^{+} neutrophils (N+/+ and N−/−) analyzed by a microarray.

(B and C) MPO, CTSG, and PR3 mRNA levels in CD11b^{+}Ly6C^{lo}Ly6G^{+} neutrophils (N+/+ and N−/−) (B) and peritoneal neutrophils (C) measured by qPCR.

(D) Primary granule protein levels in peritoneal neutrophils from wild-type and *Jdp2*$^{-/-}$ mice.

(E) C/EBP gene family mRNA levels in peritoneal neutrophils from wild-type and *Jdp2*$^{-/-}$ mice measured by qPCR.

(F) DNA-binding activities of C/EBPα and C/EBPβ in wild-type and *Jdp2*$^{-/-}$ peritoneal neutrophils measured with a TransAM Transcription Factor Assay Kit.

(G) C/EBPα protein levels in nuclear extracts from wild-type and *Jdp2*$^{-/-}$ peritoneal neutrophils analyzed by immunoblotting.

(H) ChIP analyses with a Jdp2 Ab of lysates from wild-type and *Jdp2*$^{-/-}$ peritoneal neutrophils. C/EBPα and C/EBPβ promoter regions were detected by PCR.

(I) 293T cells were transfected with the indicated pCMV expression vectors. After anti-Jdp2 immunoprecipitation (IP), input and immunoprecipitates were analyzed by immunoblotting with C/EBPα and Jdp2 Abs.

(J) Wild-type peritoneal neutrophils were lysed. After anti-Jdp2 and control IgG IP, immunoprecipitates were analyzed by immunoblotting with a C/EBPα Ab.

(K) Luciferase assays examining the effects of Jdp2 on the transcriptional activity of C/EBPα. Error bars, SE (n = 3). *p < 0.05.

direct binding between Jdp2 and the ATF3 promoter (Figure 6F). Furthermore, we found that Jdp2 overexpression in wild-type neutrophils suppressed ATF3 promoter acetylation (Figure 6G).

Finally, to determine whether increased ATF3 expression affected neutrophil differentiation, we infected wild-type bone marrow cells with a retrovirus encoding ATF3 and GFP or GFP alone and analyzed the cells by FACS after 5 days as described earlier. Intriguingly, neutrophils infected with the ATF3-GFP retrovirus showed decreased Ly6G expression levels but unchanged cellular morphology, compared with GFP-alone control cells (Figure 6H). Thus, ATF3 is a negative regulator of neutrophil differentiation, and its expression is strictly regulated by Jdp2.

Jdp2$^{-/-}$ Mice Are Highly Susceptible to Bacterial and Fungal Infection

Because decreased neutrophil function is an important risk factor for *C. albicans* infection, we checked the susceptibility of *Jdp2*$^{-/-}$ mice to *C. albicans* challenge (Figures 7A–7C). We observed a slight but significant increase in *C. albicans* susceptibility in *Jdp2*$^{-/-}$

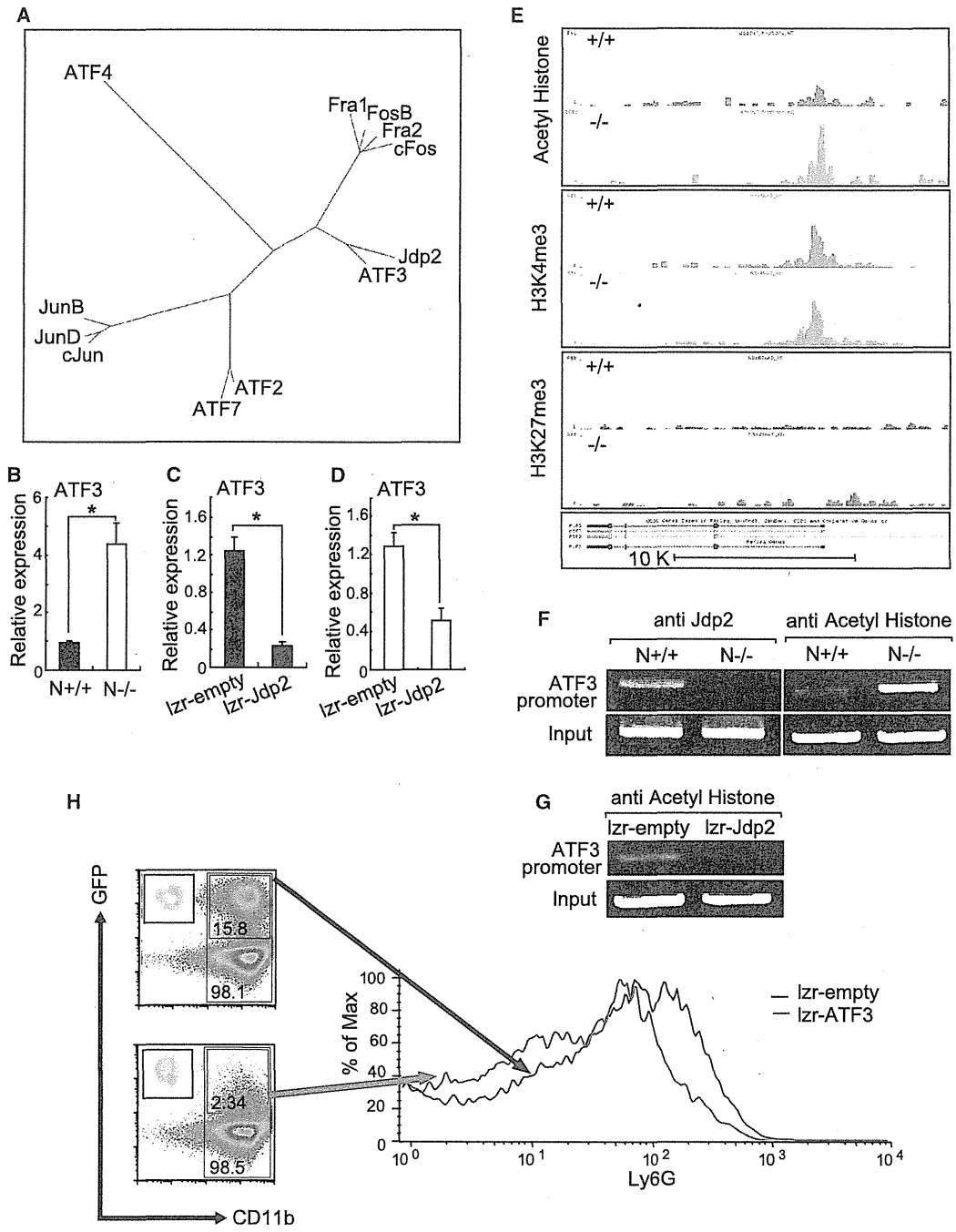

Figure 6. ATF3 Is a Target of Jdp2 and Modulates Ly6G Surface Expression

(A) Phylogenetic tree for AP-1 family proteins and Jdp2.

(B) ATF3 mRNA levels in bone marrow CD11b$^+$Ly6C^{lo}Ly6G$^+$ neutrophils (N+/+ and N−/−) analyzed by qPCR.

(C and D) Wild-type (C) and *Jdp2*$^{-/-}$ (D) bone marrow cells were infected with a retrovirus encoding Jdp2 and GFP (lzr-Jdp2) or GFP alone (lzr-empty) and cultured with G-CSF. After 5 days, the cells were harvested and CD11b$^+$GFP$^+$ cells were sorted. ATF3 mRNA levels were measured by qPCR.

(E) ChIP-seq enrichment profiles for acetyl-histone, H3K4me3, and H3K27me3 at the ATF3 locus in wild-type and *Jdp2*$^{-/-}$ peritoneal neutrophils.

(F) ChIP analyses with Jdp2 and acetyl-histone Abs of lysates from wild-type and *Jdp2*$^{-/-}$ bone marrow CD11b$^+$Ly6C^{lo}Ly6G$^+$ neutrophils (N+/+ and N−/−). DNA fragments of the ATF3 promoter region were detected by PCR.

(G) ChIP analyses with an acetyl-histone Ab of lysates in (C). DNA fragments of the ATF3 promoter region were detected by PCR.

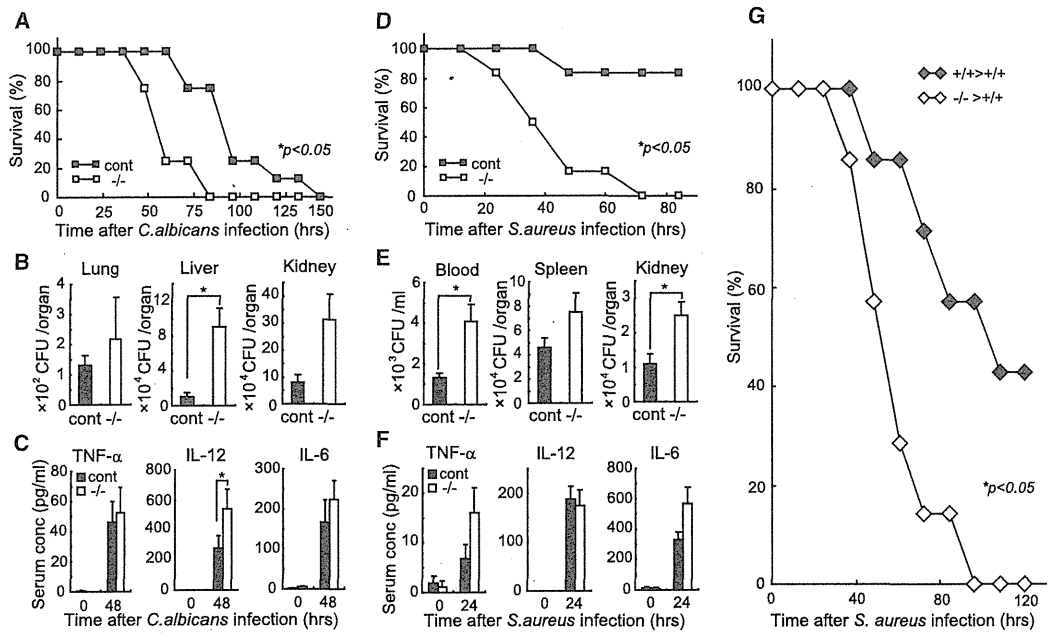

Figure 7. *Jdp2*$^{-/-}$ Mice Are Susceptible to Infection

(A–C) *C. albicans* was intravenously injected into *Jdp2*$^{+/+}$*Jdp2*$^{+/-}$ mice (control, n = 8) and *Jdp2*$^{-/-}$ mice (−/−, n = 8) and the mice were monitored (A). *Jdp2*$^{-/-}$ mice showed significantly worse survival than control mice ($p < 0.05$). CFU in the indicated organs (B) and serum cytokine levels (C) were determined at 36 hr after infection.

(D–F) *S. aureus* was intravenously injected into *Jdp2*$^{+/+}$*Jdp2*$^{+/-}$ mice (control, n = 6) and *Jdp2*$^{-/-}$ mice (−/−, n = 6) and the mice were monitored (D). *Jdp2*$^{-/-}$ mice showed significantly worse survival than control mice ($p < 0.05$). CFU in the indicated organs (E) and serum cytokine levels (F) were determined at 36 hr after infection.

(G) *S. aureus* was intravenously injected into wild-type mice reconstituted by transplantation of wild-type (+/+ > +/+, n = 7) or *Jdp2*$^{-/-}$ (−/− > +/+, n = 7) bone marrow and the mice were monitored. The survival was significantly worse in −/− > +/+ mice than in +/+ > +/+ mice ($p < 0.05$).

Error bars, SE (n = 4 unless indicated). *$p < 0.05$.

mice (Figure 7A), which showed elevated numbers of *C. albicans* CFU in their liver compared with wild-type mice (Figure 7B). The serum IL-12 levels were significantly elevated in *Jdp2*$^{-/-}$ mice, compared with wild-type mice, but the serum TNF-α and IL-6 levels were comparable (Figure 7C). We also infected *Jdp2*$^{-/-}$ and wild-type mice with *S. aureus* (Figures 7D–7F). Surprisingly, *Jdp2*$^{-/-}$ mice were highly susceptible to *S. aureus* infection, compared with wild-type mice (Figure 7D). *Jdp2*$^{-/-}$ mice showed significantly elevated numbers of *S. aureus* CFU in their blood and kidneys, compared with wild-type mice (Figure 7E). In contrast, the serum cytokine levels were not significantly altered (Figure 7F). To evaluate the relevance of hematopoietic Jdp2 deficiency to protective immunity against pathogens, we irradiated wild-type mice and reconstituted them with bone marrow from wild-type or *Jdp2*$^{-/-}$ mice (Figure 7G). Chimeric mice lacking Jdp2 in their hematopoietic system showed significantly increased susceptibility to *S. aureus* (Figure 7G). To evaluate the importance of lymphocytes in protective immunity against *S. aureus* infection in our experimental model, we depleted T and B cells in wild-type mice reconstituted with bone marrow from wild-type mice by using CD3 and CD20 Abs (Figure S6A). However, this depletion had no effect on survival in response to *S. aureus* (Figure S6B). Thus, we think our infection model reflects the function of cells other than T and B cells. Next, we depleted neutrophils in bone marrow chimeric mice by using a Ly6G Ab (Figure S6C). We infected the neutrophil-depleted chimeric mice with *S. aureus* and observed no significant difference in *S. aureus*-induced lethality between wild-type and *Jdp2*$^{-/-}$ chimeric mice (Figure S6D). Thus, these findings suggest that the increased susceptibility to *S. aureus* in *Jdp2*$^{-/-}$ mice is due to an abnormal neutrophil phenotype.

DISCUSSION

We have demonstrated that Jdp2 plays a critical role in osteoclastogenesis in vivo. We also discovered that in vitro osteoclastogenesis was completely abolished in *Jdp2*$^{-/-}$ cells. Furthermore, RANKL-mediated Jdp2 induction appeared to be

(H) Wild-type bone marrow cells were infected with a retrovirus encoding ATF3 and GFP (Izr-ATF3) or GFP alone (Izr-empty) and cultured with G-CSF. After 5 days, CD11b^{+}GFP^{+} cells were gated and Ly6G expression levels were quantified by FACS. Gated cells were also sorted and stained by May-Grunwald-Giemsa (upper left insets in the scatter plots).

Error bars, SE (n = 3). *$p < 0.05$.

regulated through c-Fos. NFATc1 activation was partially suppressed, meaning that Jdp2 may also positively regulate its activity. However, we did not detect a direct association between Jdp2 and NFATc1 (data not shown), and Jdp2 had no effect on NFATc1 binding to its promoter region. Thus, indirect mechanisms are likely to modulate the NFATc1 activity.

The defect in osteoclastogenesis in *Jdp2*$^{-/-}$ mice in vivo was relatively mild compared with its effect on in vitro osteoclastogenesis. It has been reported that calcium signaling is important for NFATc1 activation and that such costimulatory signaling is supported by ITAM-harboring adaptors, such as FcRγ and DNAX-activation protein 12 (DAP12) (Koga et al., 2004). Because RANKL-induced calcium oscillation was normal in *Jdp2*$^{-/-}$ cells in this study, signaling through ITAM-harboring molecules is likely to be normal. These findings further suggest that another unknown costimulatory signaling pathway may be compensating for the Jdp2 deficiency in vivo. Further studies are needed to explore the role of Jdp2 in osteoclasts, but our data provide insights into c-Fos-Jdp2 axis-mediated osteoclastogenesis and suggest a basis for the possibility of Jdp2-targeted therapeutic approaches to treat osteoporosis.

Our present data clearly revealed an unexpected and strict requirement for Jdp2 in proper differentiation of neutrophils. *Jdp2*$^{-/-}$ neutrophils were morphologically normal but had impaired surface expression of Ly6G, apoptosis, and bactericidal function. Furthermore, C/EBPα activation and expression of Bcl-2 and primary granule genes were increased. Our data also suggest that Jdp2 suppresses C/EBPα by directly binding to it. Notably, *Jdp2*$^{-/-}$ neutrophils showed normal primary granule protein levels but increased mRNA levels. Similar discrepancies have been reported in several knockout mice. For example, Gfi1-deficient neutrophils exhibit immature morphology and significant increases in mRNAs, such as primary granule genes and C/EBPα, but lack granules (Hock et al., 2003). Meanwhile, Ikaros-deficient neutrophils have impaired Ly6G levels but normal granule and nuclear morphology, whereas secondary granule mRNAs seem to be increased (Dumortier et al., 2003). Thus, increased mRNA levels of granule genes can be considered a leading indicator for obstruction of differentiation. Unfortunately, we cannot explain why the granule mRNA and protein levels are dissociated in neutrophils. One hypothetical explanation is that there are insufficient amounts of translational components for granule synthesis in *Jdp2*$^{-/-}$ neutrophils.

Jdp2 is homologous to ATF3, a negative regulator of TLR signaling, and ATF3 expression is suppressed by Jdp2 in fibroblasts (Weidenfeld-Baranboim et al., 2009). Our genome-wide analysis revealed that the ATF3 promoter region was highly acetylated in *Jdp2*$^{-/-}$ neutrophils. Furthermore, we discovered that Jdp2 directly binds to the ATF3 promoter in neutrophils. Importantly, ATF3 may function as a novel negative regulator of Ly6G. We also revealed that *Jdp2*$^{-/-}$ mice are highly susceptible to infection. The impaired NET formation and ROS production in *Jdp2*$^{-/-}$ neutrophils may be responsible for the increased susceptibility to infection in *Jdp2*$^{-/-}$ mice. Intriguingly, this defect in the in vitro bactericidal function of *Jdp2*$^{-/-}$ neutrophils was mild compared with the highly impaired resistance to *S. aureus* infection. We cannot completely exclude the possibility of defects in other immune cells. Thus, our data suggest the importance of Jdp2 in host defense and also enhance curiosity to clarify the importance of Jdp2 in a wide range of hematopoietic cell functions.

Taken together, we have identified Jdp2 as a critical "osteo-innate-immunological" regulator both in vivo and in vitro. Thus, Jdp2-mediated gene regulation may be a critical target for the development of therapeutics to control abnormal neutrophil- and osteoclast-associated diseases.

EXPERIMENTAL PROCEDURES

Mice, Cells, and Reagents

The generation of *Jdp2*$^{-/-}$ mice is described in the Supplemental Experimental Procedures. Mice were housed in specific-pathogen-free conditions and all animal experiments were carried out with the approval of the animal research committee of the Research Institute for Microbial Diseases (Osaka University). Peritoneal neutrophils were prepared as described (Bertram et al., 2012). B and T cells were isolated from splenocytes with anti-B220 and anti-Thy-1.2 magnetic beads (Miltenyi Biotec), respectively. Splenic dendritic cells (DCs) were isolated with anti-CD11c magnetic beads (Miltenyi Biotec). Primary osteoclasts are prepared as described (Takegahara et al., 2006). Splenic- or bone marrow-derived $CD11b^+F4/80^+$ macrophages and $CD11b^+Ly6C^{lo}Ly6G^+$ neutrophils were sorted with a FACSAria (BD Biosciences). Conventional dendritic cells (cDCs) were prepared as described (Kato et al., 2005). *S. aureus* 834 was kindly provided by A. Nakane (Hirosaki University, School of Medicine, Aomori, Japan). This strain was cultured on tryptic soy broth agar plates at 37°C for 24 hr before use. *C. albicans* THK519 was obtained from a patient admitted to Tohoku University Hospital (Sendai, Japan). These cells were cultured on potato dextrose agar (PDA) plates (Eiken) at 30°C for 72 hr before use. The pathogen-associated molecular patterns (PAMPs), Abs, and ELISA kits listed in the Supplemental Experimental Procedures were purchased. Phagocytosis was quantified with a phagocytosis assay kit (500290; Cayman Chemical Company). Superoxide and apoptosis levels were measured with a Diogenes Cellular Luminescence Enhancement System (National Diagnostics) and annexin V-indocarbocyanine (BioVision), respectively.

Analysis of Osteoclastogenesis and Bone Phenotype

For in vitro osteoclast culture, MDMs were generated as described (Maruyama et al., 2006). MDMs were induced to differentiate into osteoclasts in the presence of 25 ng/ml M-CSF and various concentrations of RANKL (R&D Systems). After 3 days, TRAP staining was performed as described (Zhao et al., 2006). For pit assays, MDMs were cultured on bone resorption assay plates (Iwai Chemical Company) with 50 ng/ml RANKL. After 5 days, the plates were immersed in 1 M NH_4OH for 3 hr, and the resorption pits were counted. The in vivo bone phenotype was analyzed as described in the Supplemental Experimental Procedures.

Bone Marrow Transfer

Bone marrow transplantation was performed as described in the Supplemental Experimental Procedures.

qPCR

RNA was extracted from cells with TRIzol (Invitrogen Life Science Technologies), and reverse transcription was performed with ReverTra Ace (Toyobo Co. Ltd.). qPCR was performed in an ABI PRISM 7500 with TaqMan Assay-on-demand primers (Applied Biosystems).

Viral Gene Transfer and RNA Interference

Retroviral or lentiviral gene transfer was performed as described in the Supplemental Experimental Procedures. A siRNA for c-Fos (Stealth RNAi siRNA, MSS247212; Invitrogen) and a control oligo (Stealth RNAi siRNA, negative control Med GC; Invitrogen) were introduced into MDMs with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols.

Immunoblotting and Immunoprecipitation

Immunoblotting and immunoprecipitation were performed as described (Kawagoe et al., 2009).