Colonic tissue samples were fixed in 4% buffered paraformaldehyde, embedded in O.C.T. compound and cryosectioned. Sections were incubated first with proteinase K (20 μg/ml) for 15 min at 37°C, then with TdTase and biotin 14-ATP for 1 h at 37°C and finally with Alexa Fluor 488 conjugated with streptavidin and DAPI (5 μg/ml) for 2 h. Samples were mounted with VECTASHIELD and inspected with the aid of a fluorescence microscope (Olympus BX51).

Flow Cytometric Analysis of ROS Production and Measurement of Intracellular Antioxidant Activity

Flow cytometry was performed on a FACSCalibur cell sorter (Becton Dickinson), as described.[40] Briefly cells were incubated with 20 mM H_2DCF in the dark at 37°C for 30 min. The shift in green fluorescence is associated with ROS production and was determined from histogram data using CellQuest software (Becton Dickinson). A total of 20,000 cells were collected for each histogram.

To assess the antioxidant capacity in HCT-15 cells, antioxidant assay was performed using the antioxidant assay kit from Cayman chemical following the manufacturer's protocol.

Determination of the ROS-production In vivo by Electron Spin Resonance (ESR) Analysis

In vivo ESR analysis was performed as described,[41,42] with some modifications. After DSS-administration for 5 days, animals were placed under deep anaesthesia with chloral hydrate (250 mg/kg) and injected with POBN intraperitoneally (4 mmol/kg). After 1 h, mice were sacrificed, the colons were dissected and lipid phase from samples were extracted as described.[41,42] After evaporating the sample, ESR spectra were immediately recorded at room temperature using a quartz flat cell (160 μl) in a JEOL JES-TE200 spectrometer (JEOL). Operating conditions of ESR; 9.43 GHz, field 335.2 ± 5 mT, 40 mW microwave power, 100 kHz modulation frequency, 0.25 field modulation width, 0.3 ms time count and sweep time 2 min.

Statistical Analysis

All values are expressed as the mean ± standard error of the mean (S.E.M.). Two-way analysis of variance (ANOVA) followed by the Tukey test or the Student's *t*-test for unpaired results was used to evaluate differences between more than three groups or between two groups, respectively. Differences were considered to be significant for values of $P<0.05$.

Results

A Phenotype of CHOP-null Mice Resistant to Experimental Colitis

The severity of DSS-induced colitis can be monitored by various indices, such as body weight, DAI, length of colon, MPO activity, the amount of TBARS, and histological indices. We compared the development of colitis induced by 3% DSS administration in CHOP-null mice and wild-type mice by measuring body weight and DAI daily. Administration of 3% DSS caused a decrease in body weight and an increase in the DAI of the wild-type mice (Fig. 1A, B). DSS-induced colitis in this phenotype was significantly ameliorated in CHOP-null mice (Fig. 1A, B). DSS-induced colon shortening, used as a morphometric measure for the degree of inflammation, was less severe in CHOP-null mice than in the wild-type mice (Fig. 1C). Colonic MPO activity, an indicator of infiltration of leukocytes, was lower in DSS-administered CHOP-null mice than in the wild-type mice (Fig. 1D). Colonic TBARS, an index of lipid peroxidation associated with inflammation, was also lower in DSS-administered CHOP-null mice than in the wild-type mice (Fig. 1E). Absence of the *Chop* gene did not

affect the background levels of these indices (colon length, colonic MPO activity and colonic TBARS) (Fig. 1C - E). Figure 1F shows results of histological analyses of colonic tissues prepared from DSS-administered and control CHOP-null mice and wild-type mice. Crypt loss and infiltration of leukocytes were more apparent in sections from DSS-administered wild-type mice than those from CHOP-null mice. Histologic score analysis (damage score and extent of lesion) revealed that the histological differences between DSS-administered CHOP-null mice and the wild-type mice were statistically significant (Fig. 1G and H). Results in Fig. 1 show that CHOP-null mice are more resistant to DSS-induced colitis than the wild-type mice.

We examined the effect of DSS-administration on expression of CHOP and GRP78 in the intestine at both mRNA and protein levels. Analysis by real-time RT-PCR revealed that the mRNA expression of *Chop* in colonic tissues was induced by the DSS-administration in the wild-type mice and that the *Chop* mRNA was not expressed in CHOP-null mice (Fig. 2A). The mRNA expression of *Grp78* was also induced by DSS-administration (Fig. 2A), suggesting that the ER stress response is induced

simultaneously with development of DSS-induced colitis. Results in Fig. 2A also suggest that CHOP positively regulates the mRNA expression of *Grp78* in the intestine.

Immunohistochemical and immunoblotting analyses demonstrated that DSS-administration increases the level of CHOP in colonic tissues in the wild-type mice but not in CHOP-null mice (Fig. 2B and D). CHOP expression was localized in nuclei, being consistent that CHOP is a transcription factor. We also found that DSS-administration increases the level of GRP78 in colonic tissues in wild-type mice and that this increase was not so clearcut in CHOP-null mice (Fig. 2C and D).

We also examined the effect of a deficiency in CHOP on the development of TNBS-induced colitis. As shown in Fig. 3A, a TNBS (3 mg/kg)-dependent decrease in body weight was less apparent in CHOP-null mice than in wild-type mice. Administration of a higher dose of TNBS (8 mg/kg) caused the death of some mice, with the survival rate of CHOP-null mice being much higher than that of wild-type mice (Fig. 3B). Histological analysis of colonic tissues revealed that TNBS-dependent intestinal mucosal damage was more severe in the wild-type mice than in CHOP-null mice (Fig. 3C

- E). We also found that this TNBS (3 mg/kg) administration up-regulated the mRNA expression of *Chop* and *Grp78* in the intestine (data not shown).

Analysis of the mRNA Expression of Various Genes in DSS-Administered CHOP-null Mice and Wild-type Mice.

To obtain a better understanding of the molecular mechanism governing CHOP-dependent exacerbation of DSS-induced colitis, we compared the mRNA expression of various genes in the intestine of both DSS-administered CHOP-null mice and wild-type mice. Tested genes included those for pro-inflammatory cytokines (*Il-1β*, *Tnf-α* and *Il-6*), CAMs expressed on vascular endothelial cells (*Madcam-1*, *Vcam-1* and *Icam-1*), CAMs expressed on leukocytes (*CD11b*, *CD49d* and *L-selectin*), an anti-inflammatory cytokine (*Il-10*), and CHOP-regulated genes (*Caspase-11*, *Ero-1α* and *Bcl-2*). Mac-1 and very late gene (VLA)-4 are CAMs expressed on leukocytes and Mac-1 is a heterodimer of CD11b and CD18 and VLA-4 is a heterodimer of CD49d and CD29. The mRNA expression of all of the pro-inflammatory cytokine genes tested was up-regulated by DSS-administration, while the mRNA expression of *Tnf-α* but not that of

Il-1β and *Il-6* was significantly lower in DSS-administered CHOP-null mice than in wild-type mice (Fig. 4A). On the other hand, the mRNA expression of *Il-10* was higher in DSS-administered CHOP-null mice than in wild-type mice (Fig. 4D).

The mRNA expression of all CAM genes tested was also up-regulated by DSS-administration and the mRNA expression of *Vcam-1* and *CD11b* but not other CAM genes was significantly lower in DSS-administered CHOP-null mice than in wild-type mice (Fig. 4B and C).

The mRNA expression of *Caspase-11* and *Ero-1α* but not *Bcl-2* was up-regulated by DSS-administration in the wild-type mice, and the mRNA expression of *Caspase-11* and *Ero-1α* was significantly lower in DSS-administered CHOP-null mice than in wild-type mice (Fig. 4E and F).

The results in Fig. 4 suggest that CHOP regulates the mRNA expression of *Tnf-α*, *Il-10*, *CD11b*, *Caspase-11* and *Ero-1α* under inflammatory conditions. To test this idea *in vitro*, we compared the mRNA expression of these factors in the presence of LPS in peritoneal macrophages prepared from CHOP-null mice and wild-type mice. As shown in Fig. 5A, LPS-treatment stimulated the expression of *Chop* and *Grp78* mRNAs in

macrophages in a CHOP-dependent manner. The mRNA expression of *CD11b, Caspase-11* and *Ero-1α* was up-regulated by the LPS-treatment, and the expression of these genes was significantly lower in LPS-treated CHOP-null macrophages than in the wild-type macrophages (Fig. 5C, E and F). The mRNA expression of *Il-10* in the presence of LPS was higher in CHOP-null macrophages than in wild-type macrophages. However, no significant difference was found in the mRNA expression of *Tnf-α* (Fig. 5B and D). These results suggest that CHOP is involved in the expression of *CD11b, Caspase-11, Il-10* and *Ero-1α* under inflammatory conditions, and that these may play an important role in CHOP-dependent exacerbation of DSS-induced colitis.

Involvement of IL-1β and Infiltration of Macrophages in the CHOP-dependent exacerbation of DSS-induced colitis.

CHOP-induced expression of Caspase-11 and the resulting activation of Caspase-1 and stimulation of production of IL-1β are responsible for CHOP-dependent exacerbation of LPS-induced lung inflammation.[39] To test whether a similar mechanism is involved in the CHOP-dependent exacerbation of DSS-induced colitis, we measured

the levels of IL-1β and caspase-1 activity in colonic tissues in DSS-administered mice. As shown in Fig. 6A and B, DSS-administration increased the level of IL-1β and caspase-1 activity in colonic tissues in wild-type mice. These alterations were significantly suppressed in CHOP-null mice. Similar results were observed in LPS-treated macrophages; the levels of IL-1β and the caspase-1 activity in LPS-stimulated peritoneal macrophages prepared from CHOP-null mice were lower than in those from the wild-type mice (Fig. 6C and D).

Mac-1 is a CAM expressed on macrophages; its binding to ICAM-1 expressed on vascular endothelial cells is important for infiltration of blood-circulating macrophages into inflamed tissues.[43] To test whether infiltration of macrophages is involved in the CHOP-dependent exacerbation of DSS-induced colitis, we compared infiltration of macrophages between DSS-administered CHOP-null mice and the wild-type mice by immunohistochemical analysis with an antibody against CD68, which is expressed on lysosomal membranes of macrophages.[44] As shown in Fig. 7A (see magnified panels), the antibody targeted mononuclear cells, confirming that this antibody can be used to detect macrophages. Results in Fig. 7A show that DSS-induced infiltration of macrophages into

colonic tissues was suppressed in CHOP-null mice compared to the wild-type mice. To confirm a role for CHOP in the expression of *CD11b* (Mac-1) *in vitro*, we used RAW264 cells (mouse leukemic monocyte) and plasmids for overexpression of CHOP and C/EBP-β, known to act co-ordinately with CHOP in the transcription of some genes.[45] As shown in Fig. 7B, *CD11b* mRNA expression was up-regulated by transfection of cells with the plasmid with *Chop*, and this up-regulation was further stimulated by simultaneous transfection with a plasmid with *C/ebp-β*. We confirmed the overexpression of *Chop* and *C/ebp-β*, depending on the transfection of each plasmid (Fig. 7C and D). We found the CHOP-binding consensus sequences in the promoter of *CD11b* (Fig. 7E). Overexpression of *C/ebp-β* did not affect the mRNA expression of *Chop*. Results in Fig. 7 suggest that up-regulation of the expression of *CD11b* (Mac-1) by CHOP is involved in CHOP-dependent exacerbation of DSS-induced colitis through stimulation of macrophage infiltration into the intestine.

Involvement of ROS-production and ROS-induced Apoptosis in Exacerbation of DSS-induced Colitis

We measured ROS-production in the intestine by measuring the lipid-derived free radical spin adduct with ESR spectroscopy and spin trap POBN, which reacts with ROS to form a radical spin adduct. This method, *in vivo* free radical production *ex vivo* detection, was shown to be effective for monitoring ROS-production in the lung *in vivo*.[41,42] However, this is the first attempt to use this technique on intestinal tissue. As shown in Fig. 8A, a radical spin adduct of ESR spectrum similar to that reported in the lung was obtained. The hyperfine coupling constants for the POBN radical adducts were $\square^N = 14.91 \pm 0.08$ G and $\square_\beta = 2.45 \pm 0.04$ G, which are similar to data previously reported in the lung,[41,42] suggesting that this ESR spectrum is derived from lipid-derived free radicals and that this method can be used for the monitoring the ROS-production at the intestine *in vivo*. As shown in Fig. 8B, the level of ROS-production in the intestine was elevated by DSS-administration in the wild-type mice and lower in DSS-administered CHOP-null mice than in the wild-type mice. We also found that administration of vitamin E, an antioxidant decreased the intestinal level of ROS-production and the DAI in DSS-treated wild-type mice (data not shown). Results

suggest that the DSS-induced production of ROS in the intestine was suppressed in CHOP-null mice.

Production of ROS in peritoneal macrophages prepared from CHOP-null mice and wild-type mice was compared using FACS analysis. The cell permeable fluorescent dye, H_2DCF, can be converted to a fluorescent product, DCF, in a ROS-dependent manner. Therefore, the increase in the green fluorescence (X axis in Fig. 8C, D, G) is indicative of an increase in the level of ROS-production.[40] Treatment of wild-type macrophages with LPS stimulated ROS-production (Fig. 8C), as has been described previously.[46] However, this stimulation was not clearly observed in CHOP-null macrophages, indicating that CHOP is responsible for LPS-stimulated ROS-production (Fig. 8C). We also examined the effect of siRNA for CHOP on LPS-stimulated ROS-production in RAW264 cells (Fig. 8D). We confirmed that treatment of RAW264 cells with LPS up-regulated the expression of *CHOP* mRNA and that transfection of cells with siRNA for CHOP significantly suppressed the expression of not only *CHOP* but also *Ero-1*□mRNA (Fig. 8E, F). As is the case with peritoneal macrophages, treatment of RAW264 cells with LPS stimulated ROS-production and transfection of cells with

siRNA for *CHOP* partially suppressed this LPS-stimulated ROS-production (Fig. 8D). In order to examine the role of ERO-1α in this CHOP-dependent production of ROS, we also examined the effect of siRNA for ERO-1α on LPS-stimulated ROS-production in RAW264 cells. We confirmed that transfection of cells with siRNA for ERO-1□significantly suppressed the expression of *Ero-1*□mRNA (Fig. 8H). Transfection of cells with siRNA for ERO-1αpartially suppressed this LPS-stimulated ROS-production (Fig. 8G). Results in Fig. 8 suggest that CHOP stimulates ROS-production in macrophages at least partly through the up-regulation of ERO-1α.

Next, we compared the level of apoptosis in the colonic mucosa of DSS-administered CHOP-null mice and wild-type mice by use of the TUNEL assay. More TUNEL-positive cells (apoptosis) were observed in the colonic mucosa of DSS-administered wild-type mice than in CHOP-null mice (Fig. 9A), suggesting that ROS-induced apoptosis associated with DSS-induced colitis is suppressed in CHOP-null mice.

To test the role of CHOP in ROS-induced apoptosis *in vitro*, we examined the effect of siRNA for CHOP on cell death induced by menadione, a superoxide anion (a

representative ROS) releasing drug, in a colonic cancer cell line (HCT-15). We confirmed that transfection of cells with siRNA for CHOP inhibited the mRNA expression of *CHOP* in both the presence and absence of menadione (Fig. 9B). As shown in Fig. 9C, treatment of cells with menadione induced cell death in a dose-dependent manner, while transfection of cells with siRNA for CHOP significantly suppressed this menadione-induced cell death. We confirmed that cell death such as that evident in Fig. 9C was mediated by apoptosis by showing that apoptotic DNA fragmentation and chromatin condensation accompanied the cell death (data not shown). The results in Fig. 9 suggest that CHOP makes colonic cells sensitive to ROS-induced apoptosis, maybe through modulation of apoptosis-inducing pathway such as down-regulation of Bcl-2 and up-regulation of Bim.[16-18] We also examined the effect of CHOP on intracellular antioxidant activity. As shown in Fig. 9D, the level of antioxidant activity that was measured by extinction of hydrogen peroxide was not affected by transfection of siRNA for CHOP. Results in Fig. 8 and 9 imply that CHOP stimulates ROS-induced apoptosis through both increasing the ROS production and modulating apoptosis-inducing pathway.

Discussion

CHOP is a transcription factor involved in the ER stress response which has been recently revealed to play an important role in various diseases, including neurodegenerative diseases, diabetes, gastric ulcer and lung inflammation.[24,39,47-50] However, its role in IBD has remained unknown. In this study, we obtained direct genetic evidence that CHOP stimulates the development of DSS- and TNBS-induced colitis, animal models of IBD, by showing that CHOP is up-regulated by DSS- or TNBS-administration and that CHOP-null mice are resistant to development of experimental colitis in these models. It was recently reported that IRE1β knockout mice are sensitive to DSS-induced colitis[51] and that up-regulation of GRP78 in IL-10 knockout mice contributes to spontaneous development of colitis through activation of NF-κB.[21] Thus, it seems that some aspects of the ER stress response are positively involved, and other aspects negatively involved, in the development of experimental colitis.

Our *in vivo* and *in vitro* analyses suggested that CHOP stimulates the development of DSS-induced colitis via several mechanisms. One of these is the caspase-11-casapse-1-IL-1β pathway, which was recently shown to play an important

role in LPS-induced lung inflammation.[39] It is well-known that IL-1β stimulates the development of IBD and experimental colitis,[9] that CHOP positively regulates the transcription of *Caspase-11*, and that Caspase-11 activates Caspase-1, and that the production of IL-1β from pro-IL-1β is catalysed by caspase-1.[39] In this study, our *in vivo* analysis showed that the intestinal level of IL-1β and the activity of caspase-1 was lower in DSS-administered CHOP-null mice than in wild-type mice. Since the production of IL-1β is stimulated under inflammatory conditions, it is possible that this decreased level of IL-1β is not a cause but a result of the amelioration of colitis in CHOP-null mice. However, we showed that up-regulation of *Caspase-11* mRNA expression and the activity of caspase-1 in the intestine following DSS-administration was suppressed in CHOP-null mice and in macrophages prepared from CHOP-null mice, compared to the respective wild-type control. These results suggest that the caspase-11-casapse-1-IL-1β pathway is involved in the CHOP-dependent exacerbation of DSS-induced colitis.

We also postulated that CHOP positively regulates the expression of *CD11b* (*Mac-1*) and that this mechanism is involved in CHOP-dependent exacerbation of DSS-induced colitis thorough stimulation of macrophage infiltration into the intestine.

Mac-1 is expressed on the macrophage cell surface and stimulates the infiltration of blood-circulating macrophages into inflamed tissues.[43] In this study, we showed that *CD11b* (*Mac-1*) mRNA expression and infiltration of macrophages into the intestine were both reduced in DSS-administered CHOP-null mice compared to wild-type mice. We also showed that up-regulation or down-regulation of expression of *Chop* stimulates or suppresses, respectively, the expression of *CD11b* (*Mac-1*). Furthermore, we found the consensus DNA sequence for CHOP-binding in the promoter region of *CD11b*. Since IL-1β was reported to stimulate infiltration of macrophages,[52] lower levels of IL-1β in DSS-administered CHOP-null mice may contribute to their lower levels of macrophage infiltration. However, it is also possible that the CHOP-dependent inductions of *CD11b* (*Mac-1*) expression is due to the increased recruitment of Mac-1-positive myeloid cells.

We showed that intestinal ROS-production is lower in DSS-administered CHOP-null mice than in wild-type mice and propose that this is one of the mechanisms governing the CHOP-dependent exacerbation of DSS-induced colitis. Although a number of *in vitro* studies have suggested that CHOP is involved in ROS-production,[40,53] this is the first evidence showing that CHOP is involved in ROS-production *in vivo*. We

revealed this by use of radical spin adduct ESR spectrum analysis. This analysis should be useful for detecting intestinal ROS-production *in vivo*. Furthermore, we suggested that the lower levels of ROS-production in CHOP-null mice are due to the lower level of expression of ERO-1α; *Ero-1α* mRNA expression in the intestine in DSS-administered CHOP-null mice and ROS-production in LPS-stimulated macrophages prepared from the mice were lower than in the respective wild-type control samples. Furthermore, we showed that siRNA for ERO-1α suppresses LPS-stimulated ROS-production in RAW264 cells.

Analysis using the TUNEL assay revealed that DSS-induced apoptosis in colonic mucosa was inhibited in CHOP-null mice. This correlates with other parameters for DSS-induced colitis. However, it was not clear whether the alteration to apoptosis is caused by or is a result of the inhibition of DSS-induced colitis. Given that transfection with siRNA for CHOP inhibited ROS-induced apoptosis *in vitro*, this result suggests that CHOP stimulates ROS-induced apoptosis, which seems to contribute to the lower level of apoptosis in the colonic mucosa and to a phenotype resistant to DSS-induced colitis as seen in CHOP-null mice. A number of mechanisms have been proposed for the

stimulation of apoptosis by CHOP, such as down-regulation of Bcl-2, translocation of BAX to mitochondoria and activation of Bim.[16,40,54] Since we showed that *Bcl-2* mRNA expression was not affected by the lack of *Chop*, other mechanisms seem to be involved. It is also possible that activation of caspase-11 is involved in the stimulation of ROS-induced apoptosis by CHOP, because caspase-11 stimulates the activation of capsase-3 and caspase-7, both of which are directly involved in the induction of apoptosis.[55]

In addition to the mechanisms described above, other mechanisms may be involved in the CHOP-dependent exacerbation of DSS-induced colitis, such as down-regulation of IL-10 and up-regulation of GRP78. IL-10 was reported to suppress development of IBD and experimental colitis,[56] while expression of GRP78 stimulates the development of DSS-induced colitis through activation of NF-κB.[21] We showed here that expression of *Il-10* or *Grp78* mRNA in the intestine of DSS-administered CHOP-null mice was higher or lower, respectively, than in the wild-type control.

Glucocorticoids, 5-aminosalicylic acid (5-ASA) and immunosuppressive drugs are currently used for the clinical treatment of IBD.[2] Although some new types of drugs,