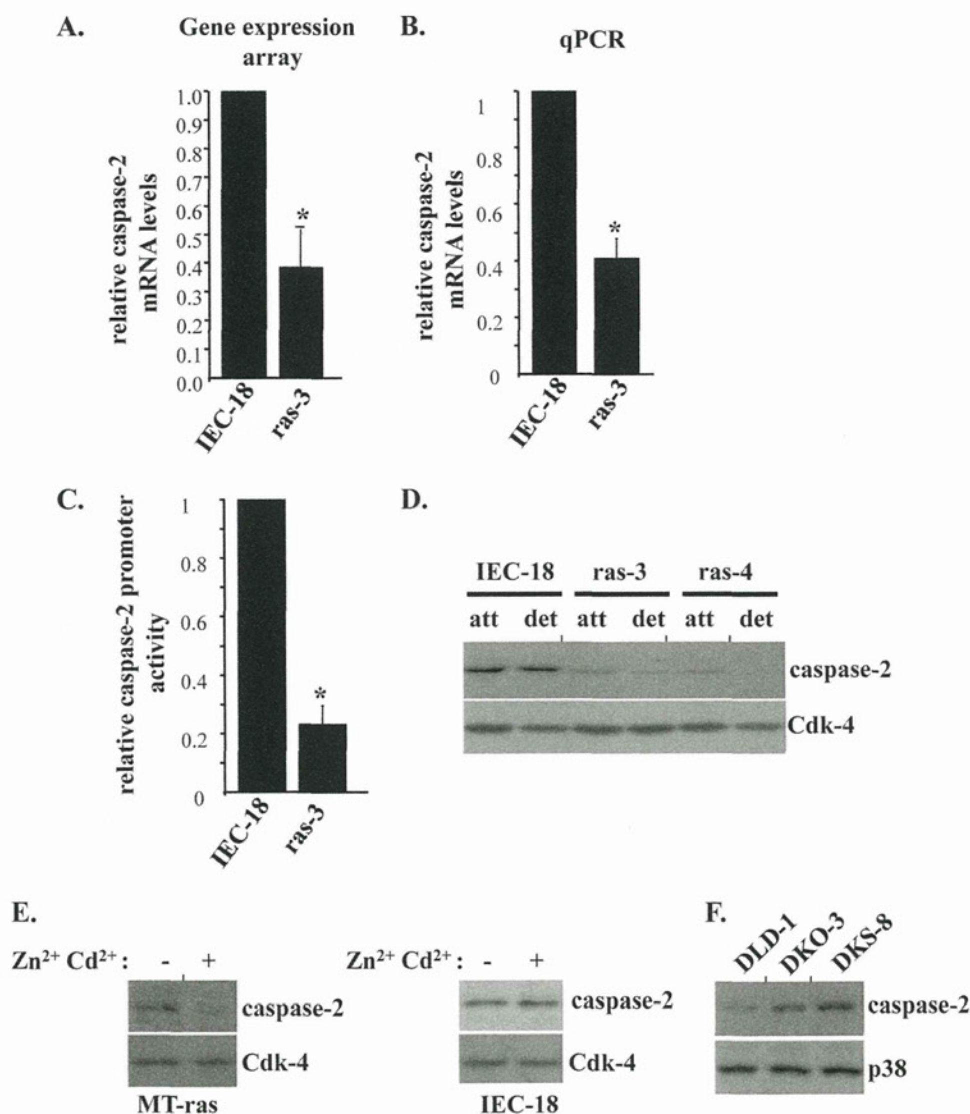

FIGURE 1. **Oncogenic *ras* down-regulates caspase-2 in intestinal epithelial cells.** *A*, IEC-18 and an H-*ras*-transformed clone of these ras-3 cells were cultured detached from the ECM for 24 h, and the expression of caspase-2 mRNA (along with other regulators of apoptosis) was analyzed in these cells by use of the rat-specific array carrying respective cDNAs (SuperArray). Signals on the array were detected by ECL. The intensity of each signal was quantified by densitometry and normalized by the levels of Ube2i mRNA that served as a loading control. The data represent the average of two independent experiments plus the S.D. *B*, indicated cell lines were analyzed for caspase-2 mRNA expression by qPCR. The observed caspase-2 mRNA levels were normalized by the levels of 18 S rRNA that were also determined by qPCR. The resulting levels of caspase-2 mRNA in IEC-18 cells were arbitrarily designated as 1.0. The data represent the average of three independent experiments plus the S.D. *C*, indicated cell lines were transiently transfected with expression vector coding for firefly luciferase under the control of the caspase-2 gene fragment containing caspase-2 promoter and an expression vector coding for *Renilla* luciferase. The intensity of respective signals corresponding to firefly luciferase activity was normalized by those of *Renilla* luciferase. The resulting numbers obtained for IEC-18 cells were arbitrarily designated as 1.0. The data represent the average of three independent experiments plus the S.D. The values observed for IEC-18 cells and marked with an *asterisk* in *A–C* were significantly ($p < 0.05$) different from those observed for ras-3 cells. *D*, IEC-18 cells and two independently derived H-*ras*-transformed clones of these ras-3 and ras-4 cells were cultured attached (*att*) to and detached (*det*) from the ECM for 24 h and assayed for the expression of caspase-2 by Western blot. *E*, attached MT-*ras* cells (*left panel*) or IEC-18 cells (*right panel*) were cultured in the absence (−) and in the presence (+) of 100 μM Zn^{2+} and 2 μM Cd^{2+} for 24 h (*left panel*) and assayed for the expression of caspase-2 by Western blot. *F*, attached human colorectal carcinoma cells DLD-1 and their K-*ras* knock-out derivatives DKO-3 and DKS-8 were assayed for the expression of caspase-2 by Western blot. The membranes in *D* and *E* were re-probed with a CDK-4 antibody and the membranes in *F* with an anti-p38 MAPK as loading controls.

has never been investigated, we decided to explore this role in this study.

We confirmed by qPCR that caspase-2 mRNA levels are significantly lower in ras-3 cells than in IEC-18 cells (Fig. 1*B*). We further found that ras-3 cells transfected with an expression vector carrying a luciferase gene under the control of a previously characterized (53) fragment of a caspase-2 gene containing a caspase-2 promoter (spanning the DNA fragment located between positions −3970 and −2595 of the caspase-2 gene) (53) displayed a noticeably lower luciferase activity than the parental IEC-18 cells transfected with the same vector (Fig. 1*C*). Collectively, the data presented above (Fig. 1, *A–C*) indicate that Ras blocks transcription of the caspase-2 gene in the intestinal epithelial cells.

We further observed that caspase-2 expression is significantly lower at the protein level in ras-3 and ras-4 (another published anoikis-resistant tumorigenic clone of IEC-18 cells) cells (1, 12) compared with the parental IEC-18 cells regardless of whether these cells were attached to or detached from the ECM (Fig. 1*D*). To confirm that the down-regulation of

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caspase-2 represents a direct consequence of the presence of oncogenic *ras* in IEC-18 cells, we utilized a published clone of IEC-18 cells MT-*ras* that harbors exogenous activated mutant of H-*ras* under the control of Zn^{2+}- and Cd^{2+}-inducible metallothionein promoter (57). We found that treatment of MT-*ras* cells with Zn^{2+} and Cd^{2+} results in a significant inhibition of caspase-2 expression (Fig. 1*E, left*). By contrast, treatment of the parental IEC-18 cells with the indicated metal ions did not cause any down-regulation of caspase-2 (Fig. 1*E, right panel*).

To establish whether Ras can promote caspase-2 down-regulation in human colon cancer cells, we utilized highly tumorigenic human colon carcinoma-derived cells DLD-1 carrying one allele of oncogenic K-*ras* and derivatives of these cells DKO-3 and DKS-8, in which the mutant K-*ras* allele had been disrupted by homologous recombination (58). We and others found previously that both oncogenic K-*ras*-deprived variants of DLD-1 cells are significantly more anoikis-susceptible (12) and much less tumorigenic (58) than oncogenic *ras*-harboring DLD-1 cells. As shown in Fig. 1*F*, we observed that DLD-1 cells carry much lower amounts of caspase-2 than the mutant K-*ras*-knock-out cells DKS-8 and DKO-3. Thus, *ras* oncogene down-regulates caspase-2 in malignant intestinal epithelial cells.

Caspase-2 Contributes to Execution of Anoikis of Intestinal Epithelial Cells—To test whether caspase-2 plays a role in the execution of anoikis of intestinal epithelial cells, we ablated this caspase in IEC-18 cells by using two separate small interfering RNAs (siRNAs) targeted to different regions of caspase-2 mRNA (Fig. 2, *A* and *D*). To assess the effect of enforced caspase-2 down-regulation on the viability of detached cells, we used a clonogenic cell survival assay that we often utilized in the past for measuring anoikis (28, 55). In the course of the assay cells transfected with control or caspase-2-specific siRNAs were cultured detached from the ECM, re-plated in a monolayer, cells that remained viable after detachment were allowed to form colonies, and the resulting colonies were counted. We found that loss of caspase-2 significantly increases the viability of IEC-18 cells following detachment from the ECM (Fig. 2, *B* and *E*). We further observed that enforced caspase-2 down-regulation noticeably reduces the ability of detached IEC-18 cells to bind annexin V (Fig. 2, *C* and *F*) (this ability is one of the well established hallmarks of apoptosis (59)). Thus, caspase-2 contributes to the execution of anoikis of nonmalignant intestinal epithelial cells.

Ras-induced Down-regulation of Caspase-2 Is Required for Anoikis Resistance of Ras-transformed Intestinal Epithelial Cells—To address the role of *ras*-induced down-regulation of caspase-2 in anoikis resistance of *ras*-transformed intestinal epithelial cells, we decided to reverse this down-regulation. To this end, we transiently transfected ras-3 (an oncogenic *ras*-expressing derivative of IEC-18 cells, see Fig. 1) with GFP-tagged caspase-2 (others established that caspase-2 remains active in the presence of the GFP tag (52)). We then isolated GFP-positive ras-3 cells by flow cytometry and confirmed that the total amount of caspase-2 in the resulting cells is significantly higher than that in the cells transfected with a control vector carrying GFP alone and does not exceed that in the parental IEC-18 cells (Fig. 3*A*). Thus, the subsequently observed effects of caspase-2 on apoptosis of ras-3 cells were

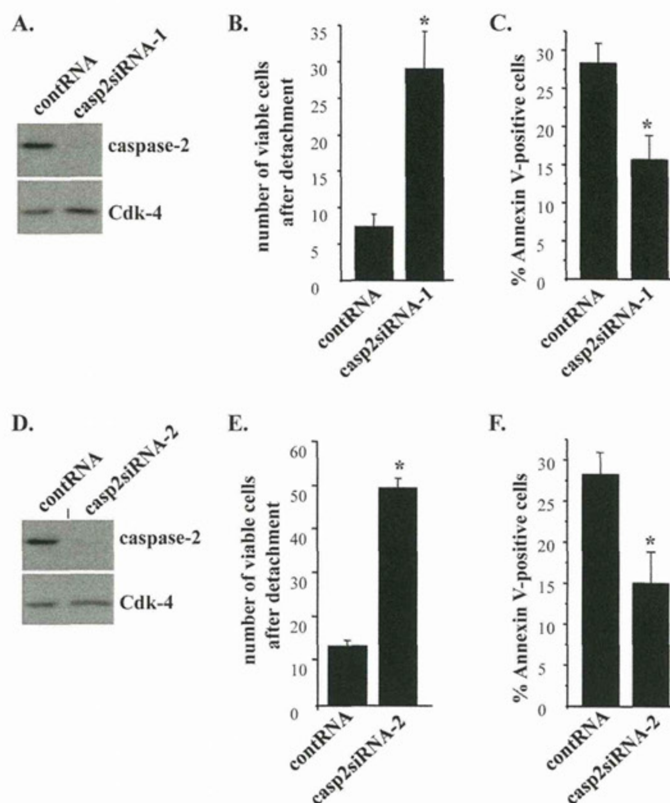

FIGURE 2. **Caspase-2 is required for anoikis of intestinal epithelial cells.** *A* and *D*, IEC-18 cells were transfected with a control RNA (*contRNA*) or caspase-2-specific siRNA1 (*casp2siRNA-1, A*) or caspase-2-specific siRNA2 (*casp2siRNA-2, D*) and assayed for caspase-2 expression by Western blot. CDK-4 was used as a loading control. *B* and *E*, cells were subsequently placed in suspension for 24 h and then re-plated in monolayer. The number of viable cells after detachment was calculated as a number of colonies formed 7 days later by the cells that survived after being cultured in suspension. The data represent the average of the triplicates plus the S.D. This experiment was repeated twice with similar results. *C* and *F*, cells transfected as in *A* and *D*, respectively, were placed in suspension for 24 h and assayed for annexin V binding by flow cytometry. The data represent the average of three experiments plus the S.E. The values marked with an *asterisk* were significantly ($p < 0.05$) different from those derived from the respective control experiments.

not due to the presence of abnormally high amounts of this caspase in the indicated cells. We then tested the ability of GFP- and caspase-2-GFP-transfected cells to bind annexin V and found that exogenous caspase-2 significantly increases the susceptibility of detached (Fig. 3*C*) but not that of the attached (Fig. 3*B*) ras-3 cells to apoptosis.

The ability of cells to grow in the absence of adhesion to the ECM as colonies in soft agar represents one of the most stringent criteria for malignant transformation that are presently being used (6, 7). We thus tested whether the reversal of the effect of oncogenic Ras on caspase-2 blocks the long term growth of the *ras* transformed in soft agar. Because transient transfection of ras-3 cells with a caspase-2 expression vector was not optimal for these type of studies, we generated three clones of ras-3 cells, ras-casp2-2, ras-casp2-3, and ras-casp2-4, expressing ectopic caspase-2 in a constitutive manner. Again, we found that the total amount of caspase-2 in each of these clones was significantly higher than that in a vector control clone (*ras*-control) but did not exceed that in the parental IEC-18 cells (Fig. 4*A*). We further observed that exogenous caspase-2 had a relatively small effect on the ability of attached

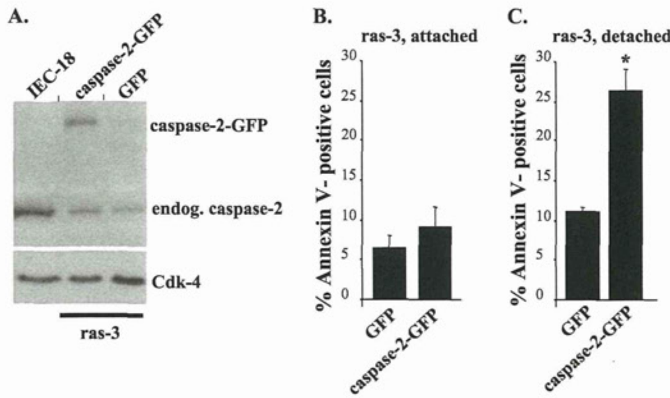

FIGURE 3. ***ras*-induced down-regulation of caspase-2 is required for anoikis resistance of malignant intestinal epithelial cells.** *A*, ras-3 cells were transfected either with a control vector carrying GFP alone or with an expression vector carrying GFP-tagged caspase-2 and assayed for caspase-2 expression along with the parental IEC-18 cells by Western blot. A caspase-2-specific antibody was used in the assay. The positions of endogenous (*endog.*) caspase-2 and exogenous GFP-tagged caspase-2 (*caspase-2-GFP*) on the gel are indicated. CDK-4 was used as a loading control. *B* and *C*, cells processed as in *A* were cultured attached to (*B*) detached from (*C*) the ECM for 48 h and analyzed for annexin V binding by flow cytometry. The data represent the average of three (*B*) and two (*C*) independent experiments plus the S.D. The value marked with an *asterisk* was significantly ($p < 0.05$) higher than that derived from the respective control experiments.

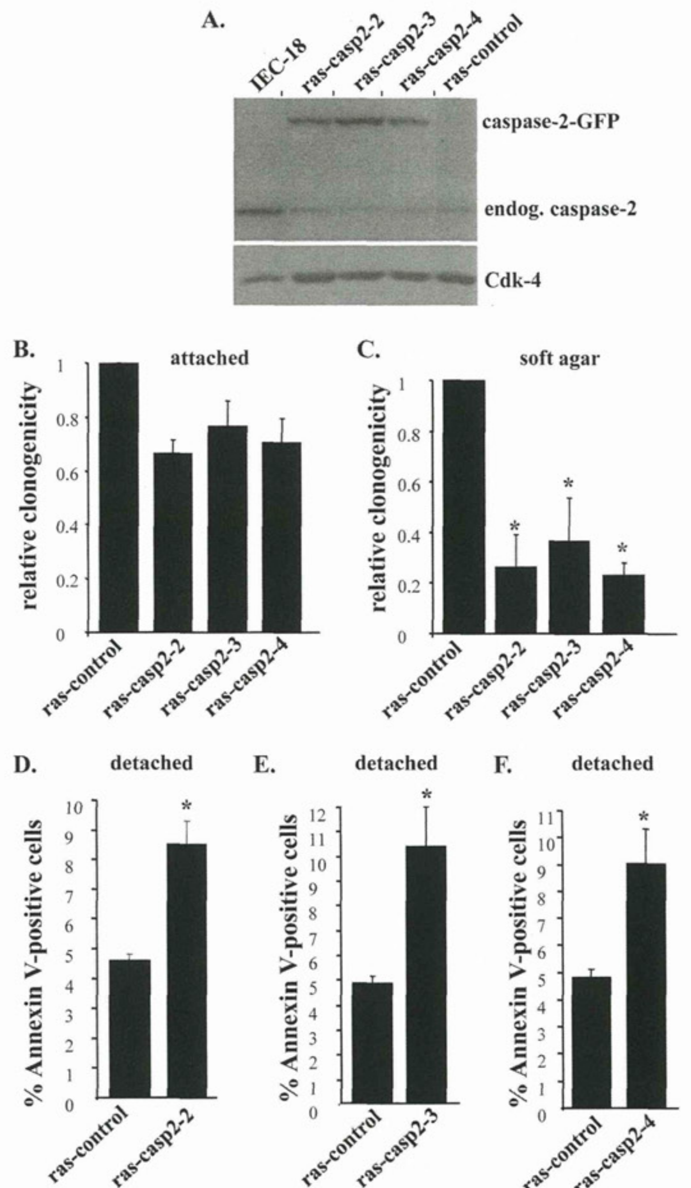

FIGURE 4. ***ras*-induced down-regulation of caspase-2 is required for the ability of malignant intestinal epithelial cells to grow in an anchorage-independent manner.** *A*, IEC-18 cells, clones of ras-3 cells ras-casp2-2, ras-casp2-3, and ras-casp2-4 (generated by transfection of ras-3 cells with a caspase-2-GFP expression vector), expressing ectopic caspase-2 in a constitutive manner and a control clone of ras-3 cells (generated by transfection of ras-3 cells with a control vector) were assayed for caspase-2 expression by Western blot. A caspase-2-specific antibody was used in the assay. The positions of endogenous (*endog.*) caspase-2 and exogenous GFP-tagged caspase-2 (*caspase-2-GFP*) on the gel are indicated. CDK-4 was used as a loading control. *B* and *C*, indicated cell lines were plated in monolayer (*B*) or in soft agar (*C*), and colonies formed by these cells were counted 7–10 days later. The number of colonies formed by the *ras*-control cells was arbitrarily designated as 1.0. The data represent the average of three independent experiments plus the S.D. *D--F*, indicated cell lines were assayed for annexin V binding by flow cytometry. The data in *D* represent the average of two independent experiments plus the S.D. The data in *E* and *F* represent the average of three independent experiments plus the S.E. Values marked with an *asterisk* were significantly ($p < 0.05$) different from those derived from the respective control experiments.

cells to form colonies (Fig. 4*B*) but noticeably blocked their clonogenicity in soft agar, when these cells were detached from the ECM (Fig. 4*C*). As expected, ectopic caspase-2, when expressed in ras-3 cells in a constitutive manner, also significantly increased their ability to bind annexin V following detachment (Fig. 4, *D–F*). Based on the data presented above, we concluded that *ras*-induced down-regulation of caspase-2 is required for the ability of oncogenic Ras to protect intestinal epithelial cells from anoikis.

We found previously that one of the mechanisms of anoikis of intestinal epithelial cells, including IEC-18 cells, is mediated by the Fas ligand, a pro-apoptotic protein that exerts its effect on cells via an adapter molecule FADD (22). We observed in this study that ablation of FADD (Fig. 5*A*) in ras-casp2-4 cells (a clone of ras-3 cells expressing ectopic caspase-2, see Fig. 4) did not block their apoptosis following detachment (Fig. 5, *B* and *C*). Thus, the pro-apoptotic signaling pathway driven by Fas ligand and FADD does not appear to be required for the ability of caspase-2 to induce anoikis of *ras*-transformed intestinal epithelial cells.

Ras-induced Down-regulation of Caspase-2 Prevents the Release of Mitochondrial Apoptosis-inducing Proteins into the Cytoplasm of Intestinal Epithelial Cells following Detachment— Molecular events involved in caspase-2-initiated apoptosis are not well understood (45). It is, however, thought that one way by which this initiator caspase promotes cell death is via facilitating (through yet unknown mechanisms) the release of the pro-apoptotic mitochondrial factors into the cytoplasm, where they activate various elements of the cellular pro-apoptotic program (47–50). We found previously in this regard that detachment of intestinal epithelial cells (including IEC-18 cells) promotes the release of the mitochondrial proteins, such as cytochrome *c* and Omi/HtrA2 in the cytoplasm and that oncogenic Ras inhibits these events (28). Furthermore, we demonstrated that Ras-induced inhibition of detachment-induced release of the mitochondrial proteins, such as Omi, is required for the ability of oncogenic Ras to suppress anoikis (28). We thus tested whether the reversal of *ras*-induced down-regula-

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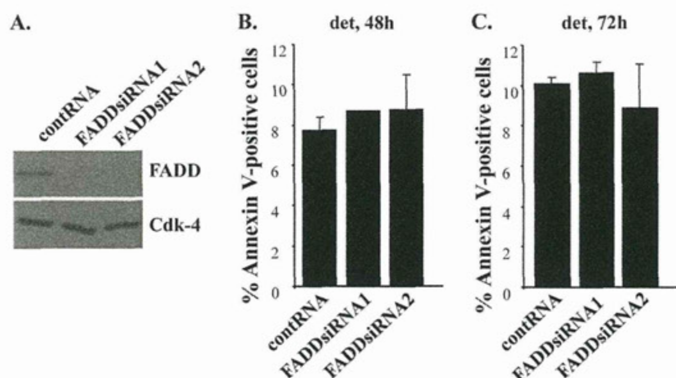

FIGURE 5. **Apoptosis of detached (*det*) *ras*-transformed intestinal epithelial cells expressing exogenous caspase-2 cannot be blocked by the ablation of FADD.** ras-casp2-4 cells (a clone of ras-3 cells expressing exogenous caspase-2) were transfected with a control RNA (*contRNA*) or FADD-specific siRNA1 (*FADDsiRNA-1*) or FADD-specific siRNA2 (*FADDsiRNA-2*) and assayed for FADD expression by Western blot. CDK-4 was used as a loading control. Cells transfected as in *A* were placed in suspension for 48 h (*B*) or 72 h (*C*) and assayed for annexin V binding by flow cytometry. The data represent the average of two independent experiments plus the S.D.

tion of caspase-2 contributes to the ability of Ras to block the release of cytochrome *c* and Omi into the cytoplasm of detached cells. As shown in Fig. 6, *A* and *B*, detached cells ras-casp2-2, ras-casp2-3, and ras-casp2-4 expressing ectopic caspase-2 displayed significantly higher amounts of both cytochrome *c* and Omi in the cytoplasm than the respective control clone *ras*-control. In agreement with a notion that caspase-2 can mediate the release of the indicated pro-apoptotic factors in detached cells, we found that ablation of caspase-2 in the parental IEC-18 cells by RNAi (see Fig. 2) blocks such release (Fig. 6, *C* and *D*). In summary, our data indicate that *ras*-induced down-regulation of caspase-2 prevents the release of cytochrome *c* and Omi into the cytoplasm of intestinal epithelial cells following their detachment.

Activity of Protein Kinase Mek Is Required for Ras-induced Down-regulation of Caspase-2 in Intestinal Epithelial Cells—Ras is known to be able to activate numerous signaling pathways, including those mediated by the sequential induction of protein kinases Raf, Mek, and Erk (40). In an effort to identify the signaling mechanism by which Ras down-regulates caspase-2 in intestinal epithelial cells, we found that treatment of ras-3 cells with PD98059, a specific and widely used small molecule inhibitor of Mek (60), resulted in a significant increase of the caspase-2 promoter activity (Fig. 7*A*), a noticeable up-regulation of caspase-2 mRNA (Fig. 7*B*), and that of caspase-2 protein (Fig. 7*C*). By contrast, treatment with LY294002, an inhibitor of phosphoinositide 3-OH kinase (61), another major mediator of Ras signaling (40), did not trigger caspase-2 protein up-regulation in these cells (data not shown). We reasoned that if the effect of Ras on caspase-2 is mediated by Mek and if this effect contributes to *ras*-induced anoikis resistance of intestinal epithelial cells, then inhibitors of Mek, such as PD98059, should promote anoikis of *ras*-transformed cells. Indeed, we found that treatment with PD98059 did not result in significant apoptosis of attached ras-3 cells but caused a noticeable increase of death of these cells when they were detached from the ECM (Fig. 7*D*). Collectively, these data are consistent with a scenario,

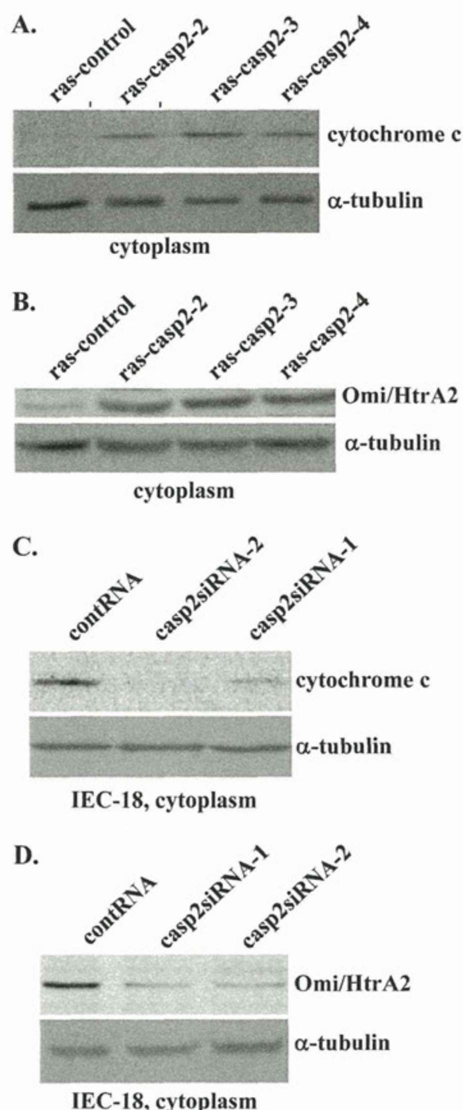

FIGURE 6. ***ras*-induced down-regulation of caspase-2 prevents the release of cytochrome *c* and Omi/HtrA2 into the cytoplasm following detachment of intestinal epithelia cells.** *A* and *B*, indicated cell lines were cultured in suspension for 24 h, and cytosolic material was isolated from these cells and assayed for the presence of cytochrome *c* (*A*) and Omi (*B*) by Western blot. *C* and *D*, IEC-18 cells were transfected as in Fig. 2 with a control RNA (*contRNA*) or caspase-2-specific siRNA1 (*casp2siRNA-1*) or caspase-2-specific siRNA2 (*casp2siRNA-2*) and cultured in suspension for 2 h; cytosolic material was isolated from these cells and assayed for the presence of cytochrome *c* (*C*) and Omi (*D*) by Western blot. α-Tubulin was used as a loading control.

according to which *ras*-induced down-regulation of caspase-2 is mediated by Mek.

Ras-induced Down-regulation of Caspase-2 Is Required for the Ability of Ras-transformed Intestinal Epithelial Cells to Form Tumors in Vivo—Normal intestinal epithelium exists *in vivo* as a single layer, whereas primary tumors as well as tumors formed by cancer cells subcutaneously injected in mice, a model that is often used for studying tumorigenesis, tend to grow as three-dimensional masses. The results of several studies, including ours, indicate that anoikis resistance of cancer cells is required for the ability of these cells to form tumors following subcutaneous injection in mice (11–15). Given that *ras*-induced down-regulation of caspase-2 is required for the ability *ras*-transformed cells to resist anoikis and grow without

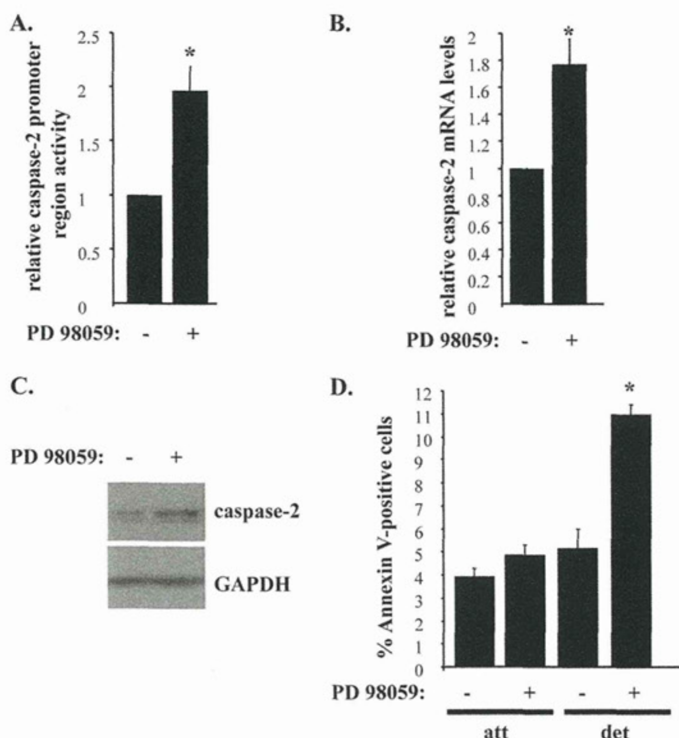

FIGURE 7. **Activity of protein kinase Mek is required for *ras*-induced down-regulation of caspase-2 in intestinal epithelial cells.** *A*, cells were transiently transfected with expression vector coding for firefly luciferase under the control of the caspase-2 gene fragment containing caspase-2 promoter, treated with either DMSO (−) or 25 μM PD98059 (+) for 24 h, and assayed for luciferase activity. The resulting numbers obtained for the untreated cells were arbitrarily designated as 1.0. The data represent the average of three independent experiments plus the S.D. *B*, ras-3 cells were treated with PD98059 as in *A* and caspase-2 mRNA levels were measured in these cells by qPCR. The observed caspase-2 mRNA levels were normalized by the levels of 18 S rRNA, which were also determined by qPCR. The resulting levels of caspase-2 mRNA in DMSO-treated cells were arbitrarily designated as 1.0. The data represent the average of three independent experiments plus the S.D. *C*, cells treated with PD98059 as in *A* were assayed for caspase-2 expression by Western blot. GAPDH was used as a loading control. *D*, cells were cultured in monolayer or suspension for 24 in the presence of either DMSO (−) or 25 μM PD98059 (+) and analyzed for annexin V binding by flow cytometry. The data represent the average of two independent experiments plus the S.D. The values marked with an *asterisk* were significantly ($p < 0.05$) different from those derived from respective control experiments.

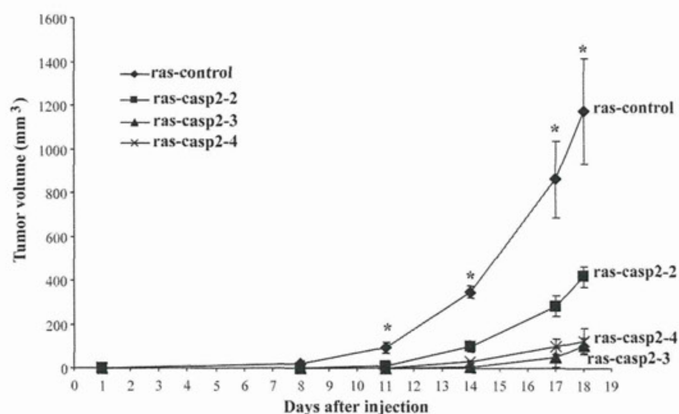

FIGURE 8. ***ras*-induced down-regulation of caspase-2 is required for the ability of malignant intestinal epithelial cells to form tumors in mice.** The indicated cell lines were injected subcutaneously into nude mice, and tumor volumes were measured at the indicated time points. Four mice were injected with each cell line. *Error bars* represent the S.E. This experiment was repeated twice with similar results. *Asterisks* indicate that values derived from the control experiments with *ras*-control cells were significantly ($p < 0.05$) higher than those observed for respective experiments with ras-casp2-2, ras-casp2-3, and ras-casp2-4 cells.

being attached to the ECM, we decided to test whether this down-regulation contributes to the ability of the indicated cells to form tumors in mice. As shown in Fig. 8, the sizes of tumors formed by cells ras-casp2-2, ras-casp2-3, and ras-casp2-4 expressing ectopic caspase-2 were significantly smaller at all times of the assay than those of tumors formed by respective control cells. Thus, *ras*-induced down-regulation of caspase-2 does contribute to the *in vivo* tumorigenicity of these cells.

In summary, we have identified a novel mechanism by which oncogenic Ras blocks anoikis of intestinal epithelial cells, allows them to grow in an anchorage-independent manner within three-dimensional multicellular masses, and enables them to form tumors. This mechanism is driven by *ras*-induced down-regulation of caspase-2.

DISCUSSION

We have identified in this study a novel mechanism by which oncogenic Ras promotes anoikis resistance of intestinal epithelial cells. This mechanism involves *ras*-dependent down-regulation of caspase-2. We found previously that anoikis of non-malignant intestinal epithelial cells is driven by detachment-induced down-regulation of Bcl-X_L (12) and subsequent release of the mitochondrial factors, such as HtrA2/Omi, into the cytoplasm (28). We have demonstrated in this study that, in addition, anoikis of these cells is mediated (via mechanisms that remain to be established) by caspase-2, a protease that according to this study (Fig. 6) and previous studies (47–50) does have the ability to increase the permeability of the mitochondria to the pro-apoptotic factors. This study as well as our previous studies indicate that oncogenic Ras has the ability to block this network of pro-anoikis signals in detached cells by activating a network of the anti-anoikis signals. We found in the past that two important elements of this network are the mechanisms involving *ras*-induced down-regulation of Bak (13) and inhibition of detachment-induced down-regulation of Bcl-X_L (12). We show here that one additional mechanism by which Ras prevents the release of cytotoxic factors, such as cytochrome *c* and Omi from the mitochondria, is driven by *ras*-dependent down-regulation of caspase-2.

Our data suggest that Ras down-regulates caspase-2 in intestinal epithelial cells by triggering a protein kinase Mek, an inducer of the MAPKs and a mediator of Ras signaling whose activity is known to be stimulated by a Ras binding partner protein kinase Raf (40). To our knowledge, this study for the first time demonstrates that the indicated signaling pathway can block anoikis downstream of Ras in intestinal epithelial cells. Of note, we observed in the past that *ras*-induced down-regulation of Bak, the second mechanism by which oncogenic Ras blocks the release of the pro-apoptotic mitochondrial factors in these cells and their subsequent anoikis, is driven by phosphoinositide 3-OH kinase (13), which represents another important mediator of Ras signaling (40). Therefore, it is the activation of both major Ras-induced signaling pathways, one mediated by Mek and another one controlled by phosphoinositide 3-OH kinase, that contributes to the indicated effects of Ras in intestinal epithelial cells.

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Interestingly, we have found that exogenous caspase-2 promoted a much more noticeable apoptosis of the *ras*-transformed cells when they were detached from the ECM than in the attached cells (see Figs. 3 and 4). These data suggest that a threshold caspase-2 concentration is required for the induction of apoptosis by signals that are induced by loss of adhesion of intestinal epithelial cells to the ECM. However, it seems likely that when caspase-2 is down-regulated in these cells (*e.g.* in response to the expression of oncogenic *ras*) signals that are induced by detachment of the indicated cells become by themselves insufficient for stimulating apoptosis.

We found in this study that *ras*-induced down-regulation of caspase-2 is required for the ability of *ras*-transformed intestinal epithelial cells to form tumors *in vivo*. These findings agree well with what is known about the role of anoikis resistance of cancer in growth of tumors formed by malignant cells. It has been well established in this regard that normal intestinal epithelium exists *in vivo* as a single layer, whereas primary human tumors as well as tumors formed by cancer cells that have been subcutaneously injected into mice, a model that we have used in this study, typically form three-dimensional masses. Perhaps not by coincidence, the ability of cancer cells to grow in a three-dimensional anchorage-independent manner in soft agar has served as a "gold standard" for malignant transformation for several decades (6), and cells that are capable of this growth can usually form subcutaneous tumors (7, 12). Moreover, treatment that blocks resistance of cancer cells to detachment-induced death is known to inhibit their ability to form such tumors (11–14). We found in the past, for example, that the reversal of *ras*-induced down-regulation of Bak (13) or the ablation of Bcl-X_L (12) in the *ras*-transformed intestinal epithelial cells enhances their susceptibility to detachment-induced death and blocks their ability to form subcutaneous tumors in mice. In addition, we found that variants of the poorly tumorigenic intestinal epithelial cells selected for increased anoikis resistance acquire the capacity for forming such tumors (18). The results of this study indicate that the reversal of *ras*-induced down-regulation of caspase-2 in malignant intestinal epithelial cells represents a relatively efficient approach for blocking growth of tumors formed by these cells.

The fact that caspase-2 can suppress anoikis and three-dimensional tumor growth is consistent with several other studies pointing at the tumor suppression function for this caspase. It was shown in this regard that mouse embryonic fibroblasts (MEFs) derived from caspase-2 knock-out mice, when transformed with oncogenes, grow faster in monolayer culture as well as in soft agar and are more tumorigenic in mice than similarly transformed wild type MEFs (62). In this case, however, loss of caspase-2 seemed to accelerate proliferation of transformed MEFs, rather than block their anoikis (caspase-2 is known to have the ability to block the cell cycle progression under certain circumstances (45)). The fact that caspase-2 mediates proliferation, rather than anoikis, of MEFs is not surprising, in view of the fact that MEFs tend not to be prone to anoikis unless they are deprived of growth factors (63, 64). It is also known that caspase-2 expression is frequently reduced in human gastric tumors when compared with normal gastric mucosa (65). Furthermore, caspase-2 was found to be significantly underexpressed in metastatic brain tumors (66). Finally, caspase-2-deficient mice were demonstrated to be noticeably more susceptible to Myc-induced lymphoma than the respective wild type mice (62).

In summary, our data indicate that the anti-apoptotic mechanism triggered by *ras*-induced down-regulation of caspase-2 represents an important novel element of the signaling network by which oncogenic Ras blocks anoikis and promotes three-dimensional growth of tumors formed by malignant intestinal epithelial cells.

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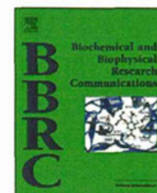

ZFAT plays critical roles in peripheral T cell homeostasis and its T cell receptor-mediated response

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ABSTRACT

ZFAT, originally identified as a candidate susceptibility gene for autoimmune thyroid disease, has been reported to be involved in apoptosis, development and primitive hematopoiesis. Zfat is highly expressed in T- and B-cells in the lymphoid tissues, however, its physiological function in the immune system remains totally unknown. Here, we generated the T cell-specific *Zfat*-deficient mice and demonstrated that *Zfat*-deficiency leads to a remarkable reduction in the number of the peripheral T cells. Intriguingly, a reduced expression of IL-7Rα and the impaired responsiveness to IL-7 for the survival were observed in the *Zfat*-deficient T cells. Furthermore, a severe defect in proliferation and increased apoptosis in the *Zfat*-deficient T cells following T cell receptor (TCR) stimulation was observed with a reduced IL-2Rα expression as well as a reduced IL-2 production. Thus, our findings reveal that Zfat is a critical regulator in peripheral T cell homeostasis and its TCR-mediated response.

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1. Introduction

We previously identified *ZFAT* (zinc-finger gene with AT-hook/zinc-finger gene in the autoimmune thyroid disease susceptibility region) as a candidate susceptibility gene for autoimmune thyroid disease [1]. *ZFAT* encodes an evolutionally conserved protein with 18 zinc-finger domains and one AT-hook domain [2], and Zfat is strongly expressed in T- and B-cells in the mouse immune-related tissues [2]. We previously reported that the *Zfat*-deficient ($Zfat^{-/-}$) mouse is embryonic lethal, and that Zfat is a critical transcriptional regulator in primitive hematopoiesis [3]. ZFAT is also involved in the regulation of apoptosis in cells of the human leukemia cell line MOLT-4 cells [4] and mouse embryonic fibroblasts [5], and in the differentiation of human umbilical vein endothelial cells [6]. Furthermore, genetic variants of *ZFAT* have been reported to be associated with adult height [7,8], with common diseases including hypertension and cancer [9,10], and with interferon-β responsiveness in multiple sclerosis [11], in which *IL-7RA* and *IL-2RA* are susceptibility genes [12–15]. Despite these advances, the function of ZFAT in the immune system remains totally unknown.

Proper regulation of peripheral T cell homeostasis is highly controlled by both cell-extrinsic and cell-intrinsic factors [16–19]. Growing evidence demonstrates that peripheral T cell homeostasis is controlled by cytokine receptor-mediated signals, especially interleukin-7 receptor (IL-7R) and interleukin-2 receptor (IL-2R) signals, as well as the interaction between TCR and major histocompatibility complex [20,21].

IL-2/IL-2R exhibits the ability to drive T cell proliferation, mediate activation-induced cell death, promote the development of regulatory T cells and modulate the expression of cytokine receptors [22,23]. The IL-2R has three chains: α, β and the common cytokine receptor γ. Resting T cells express a receptor form composed of β and γ chains that bind IL-2 with moderate affinity, whereas activation of T cells induces the α chain (a high-affinity subunit) and the formation of the high-affinity heterotrimeric receptor, which plays a critical role in T cells in the physiological context [24]. On the other hand, the IL-7R complex is composed of IL-7Rα and the γ-chain, and IL-7 signaling is mainly regulated by IL-7Rα expression in T cells [25].

Several transcriptional factors are reported to play critical roles in peripheral T cell homeostasis as cell-intrinsic factors [26–28],

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however, further studies will be needed to achieve a full understanding of the cell-intrinsic factors responsible for the integration of extrinsic signals.

Here we established and analyzed *Zfat*$^{f/f}$*-Cd4Cre* mice, revealing that Zfat is an essential molecule for peripheral T cell homeostasis and TCR-mediated cellular response.

2. Materials and methods

2.1. Mice

Zfat genomic DNA from a 129S6/SvEv^{Tac} mouse strain BAC DNA library was used to create a *Zfat*-targeting construct. A 5′ loxP site and a FRT-PGKneo-FRT-LoxP cassette were inserted into a 496-bp fragment upstream and a 376-bp fragment downstream of exon 8, respectively. A total 13-kb fragment containing 7-kb of the 5′ homology arm and 1.8-kb of the 3′ homology arm were retrieved into a plasmid vector with a diphtheria toxin cassette. The linearized targeting vector was transfected into the ES cells as described previously [3] and the G418-resistant colonies were screened by PCR. Southern blot analysis of *Eco*RI-digested genomic DNA from ES clones was performed using a 5′ external probe. The targeted ES cells were microinjected into C57BL6/NCr (Japan SLC Inc., Japan) blastocysts [3], and the resulting male chimeras were bred with C57BL/6NCr females to obtain germ-line transmission. Heterozygous offspring (*Zfat*$^{f\text{-}neo/w}$) were crossed with FLPe deleter mice from the RIKEN Bioresource centre [29] and the *Zfat*$^{f/w}$ mice were established. *Zfat*$^{f/f}$ mice were crossed with CD4-Cre mice from Taconic (Germantown, NY) to generate T-cell-specific *Zfat*- knockout, *Zfat*$^{f/f}$*-Cd4Cre* mice, in the C57BL/6 background. All the animal experiments were approved by the Animal Care and Use Committee of the NCGM Research Institute, and the experiments on mice were carried out under the guidelines of the Institutional Animal Care and Use Committee of Fukuoka University.

2.2. Antibodies

The following antibodies were purchased from BD Pharmingen (Franklin Lakes): CD4 (RM4-5), CD8 (53-6.7), B220 (RA3-6B2), CD62L (MEL-14), CD44 (IM7), TCRβ (H57-597), IL-2Rα (PC61), CD69 (H1.2F3), CCR7 (4B12), IL-7Rα (A7R34), Qa-2 (695H1-9-9), CD5 (53-7.3), HSA (LG.3A10) and CD122 (TM-β1). The antibodies used were: anti-bcl-2 (10C4) from Biolegend and anti-actin (A2066) from Sigma. Anti-ZFAT was prepared as described previously [2].

2.3. Flow cytometry

Cells from the mouse tissues were depleted of erythrocytes by hypotonic lysis. Cells were incubated with specific antibodies in the presence of 2.4G2 antibody (anti-Fcγ receptor). Data were collected with a cytometer (FACSAria II or FACSCalibur; BD Biosciences) and were analyzed with FlowJo software (Tomy Digital Biology). CFSE staining was performed by using a CellTrace CFSE Cell Proliferation Kit (Life Technologies) according to the manufacturer's instructions. Cell separation was performed with MACS Cell Separation Reagents, CD4 MicroBeads, CD8 MicroBeads and B220 (CD45R) MicroBeads (Miltenyi Biotec) or by using the cell sorting function on FACSAria II (Becton Dickinson).

2.4. Cell stimulation

To analyze cell apoptosis, naive T cells were cultured in the absence or presence of IL-7 and were stained with FITC- or APC-labeled annexin V (BD Biosciences) at the indicated time points according to the manufacturer's instructions. For plate-bound CD3/CD28 stimulation, plates were coated overnight with 1 μg ml^{-1} of anti-CD3 (145-2C11; BD Pharmingen) and 5 μg ml^{-1} of anti-CD28 (37.51; BD Pharmingen) antibodies. The amount of IL-2 was determined by using commercial ELISA kits (Invitrogen) according to the manufacturer's instructions.

2.5. Quantitative RT-PCR

Quantitative RT-PCR was performed as described previously [2,3]. The PCR primers used for each gene are described in Supplementary Table 1.

2.6. Immunoblotting

Immunoblotting was performed as described previously [2].

2.7. Statistical analysis

The data are presented as the means ± standard deviation. The statistical analyses were performed using an unpaired two-tailed Student's *t*-test. Differences at $P < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Reduction in the number of peripheral T cells in *Zfat*$^{f/f}$*-Cd4Cre* mice

To address the physiological role of Zfat in the immune system, we herein generated *Zfat*$^{f/f}$ mice, in which exon 8 of the *Zfat*

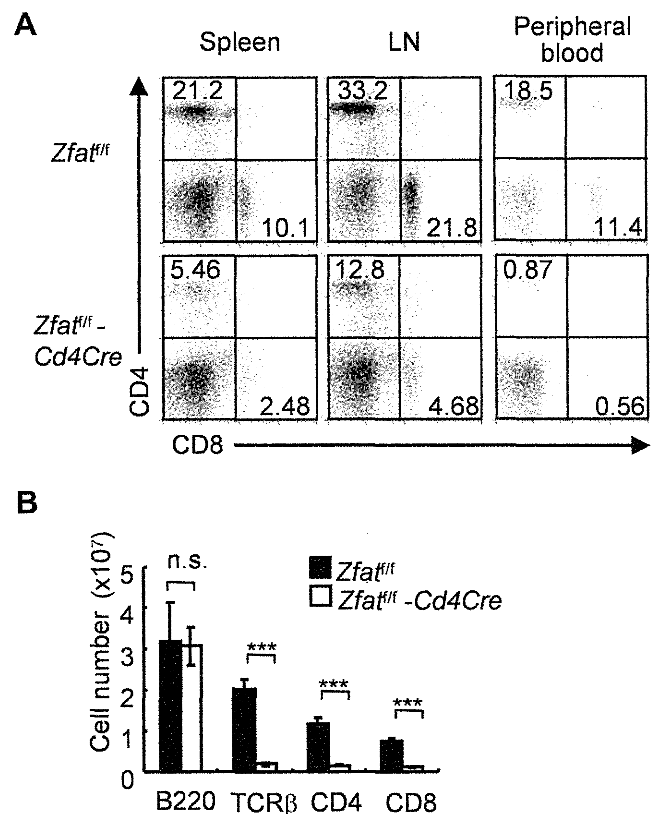

Fig. 1. Reduction in the number of peripheral T cells in the *Zfat*$^{f/f}$*-Cd4Cre* mice. (A) CD4$^+$ and CD8$^+$ cells in the spleen, lymph nodes (LN) and peripheral blood of the indicated genotypes at 8–12 weeks of age. Data are representative of three independent experiments. (B) Total number of B220$^+$, TCRβ$^+$, CD4$^+$ and CD8$^+$ subsets of the indicated genotypes at 6 weeks of age [mean ± standard deviation (s.d.), n = 4–5, $^*P < 0.0001$].

(GenBank accession No. EU221277; the same exon deleted in the $Zfat^{-/-}$ mice) was flanked by loxP sites (Supplementary Fig. 1A–C). We crossed $Zfat^{f/f}$ mice with *Cd4Cre* mice to induce a T cell-specific recombination. The efficient deletion of *Zfat* was confirmed by both PCR (Supplementary Fig. 1D) and immunoblotting of Zfat in the splenic $CD4^+$ and $CD8^+$ T cells (Supplementary Fig. 1E), thereby allowing us to study the effect of *Zfat*-deficiency in T cells *in vivo*.

We first evaluated peripheral T cells from the spleen, lymph nodes (LN) and peripheral blood. A considerable decrease in the proportions of $CD4^+$ and $CD8^+$ T cells in $Zfat^{f/f}$-*Cd4Cre* mice was observed in comparison to those in $Zfat^{f/f}$ mice (Fig. 1A). The total numbers of $CD4^+$ and $CD8^+$ T cells in the spleens of $Zfat^{f/f}$-*Cd4Cre* mice were significantly decreased by 7.4-fold and 6.6-fold, respectively, in comparison to those of $Zfat^{f/f}$ mice, whereas there were no significant differences in the number of $B220^+$ B cells (Fig. 1B), indicating that *Zfat*-deficiency results in disruption of the peripheral T cell homeostasis.

3.2. Impaired maturation of CD8SP thymocytes in $Zfat^{f/f}$-Cd4Cre mice

We examined whether the observed peripheral phenotype arises from a defect in T cell differentiation in the thymus. Analyses of the number and proportions of $CD4^-CD8^-$ double-negative (DN), $CD4^+CD8^+$ double-positive (DP), $CD4^+CD8^-$ single-positive (CD4SP) and $CD4^-CD8^+$ single-positive (CD8SP) thymic subsets and the phosphorylation of ERK induced by TCR-stimulation in thymocytes did not reveal any significant differences between the genotypes (Fig. 2A, Supplementary Fig. 2A), suggesting that obvious alterations in T cell development or TCR signaling do not occur in $Zfat^{f/f}$-*Cd4Cre* mice. Furthermore, an analysis of the expression of developmental stage-specific surface markers, including TCRβ, IL-7Rα, CD5, Qa-2 and heat-stable antigen (HSA), on DP and CD4SP cells did not reveal any differences between the genotypes (Fig. 2B and C). However, remarkable differences in the expression of CD5 and Qa-2 on CD8SP cells were observed (Fig. 2B), and the proportion of mature $TCR\beta^{hi}HSA^{lo}$ CD8SP cells in the $Zfat^{f/f}$-*Cd4Cre* mice clearly decreased in comparison to that in $Zfat^{f/f}$ mice (Fig. 2C). These results collectively suggested that *Zfat*-deficiency affects the maturation of CD8SP cells in the thymus, whereas the CD4SP cells are normally developed in the thymus of $Zfat^{f/f}$-*Cd4Cre* mice, which led us to focus on analysis of the peripheral $CD4^+$ T cells of $Zfat^{f/f}$-*Cd4Cre* mice in this study. Precise evaluation of Zfat function in T cell development in the thymus should be examined in future studies by using more sophisticated mouse models such as Lck-Cre recombinase-induced T cell-specific deletion of Zfat.

3.3. Reduced expression of IL-7Rα and impaired responses to IL-7 in peripheral $CD4^+$ T cells in $Zfat^{f/f}$-Cd4Cre mice

We next characterized the Zfat functions in peripheral $CD4^+$ T cells. No difference in the proportions of the splenic and LN naive ($TCR\beta^+CD44^{lo}$) or activated ($TCR\beta^+CD44^{hi}$) $CD4^+$ T cells between the genotypes was observed (Fig. 3A). We then assessed the homing receptors, CCR7 and CD62L (L-selectin) [26], on naive $CD4^+$ T cells in LN. In LN naive $CD4^+$ T cells from $Zfat^{f/f}$-*Cd4Cre* mice, both

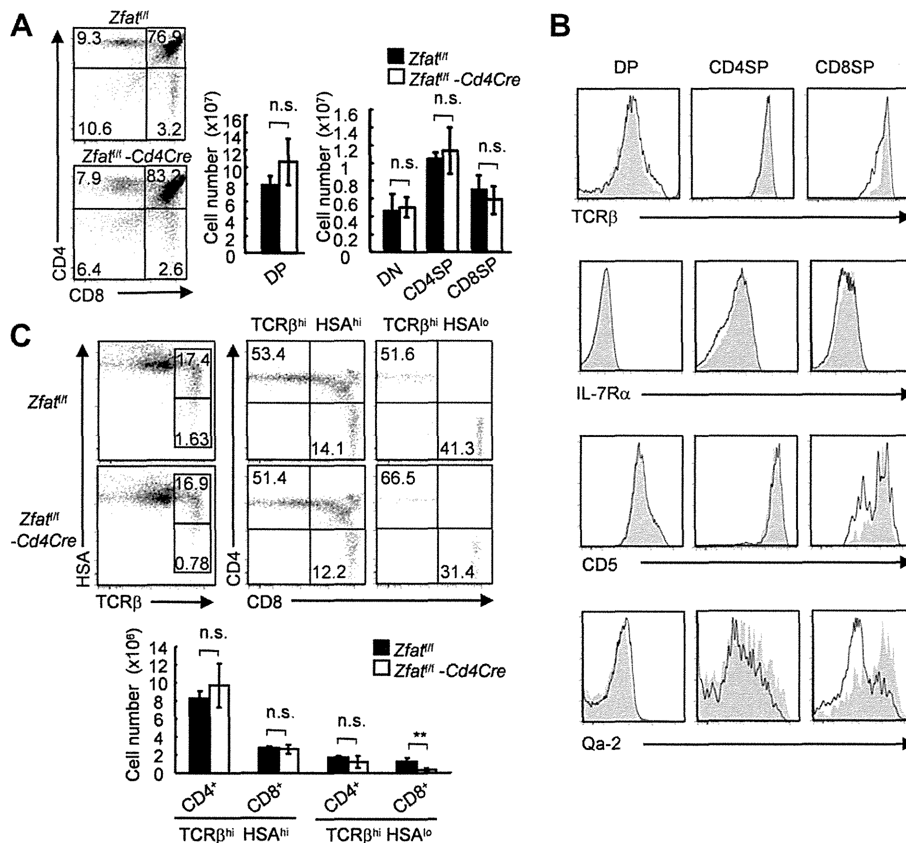

Fig. 2. Impaired maturation of CD8SP thymocytes in the $Zfat^{f/f}$-*Cd4Cre* mice. (A) Expression of CD4 and CD8 on thymocytes (left panel). Total number of thymocytes in double-positive (DP), double-negative (DN), single-positive $CD4^+$ (CD4SP) and single-positive $CD8^+$ (CD8SP) subsets at 8–12 weeks of age ($n = 4$) (right panel). (B) Expressions of TCRβ, IL-7Rα, CD5 and Qa-2 on each thymic subset from the $Zfat^{f/f}$-*Cd4Cre* (black line) and $Zfat^{f/f}$ (gray-filled) mice at 8 weeks of age. (C) TCRβ and HSA expression by thymocytes (left panel). CD4 and CD8 expression on thymocytes gated for $TCR\beta^{hi}HSA^{hi}$ and $TCR\beta^{hi}HSA^{lo}$ (right panel) at 8–12 weeks of age. Data are representative of three independent experiments. Number, proportion of the cells within each gate. The absolute numbers of mature ($TCR\beta^{hi}HSA^{lo}$) and immature ($TCR\beta^{hi}HSA^{hi}$) thymocyte subpopulations are shown. The data are the mean ± s.d.. $^{**}P < 0.05$; n.s., not significant. ($n = 3$).

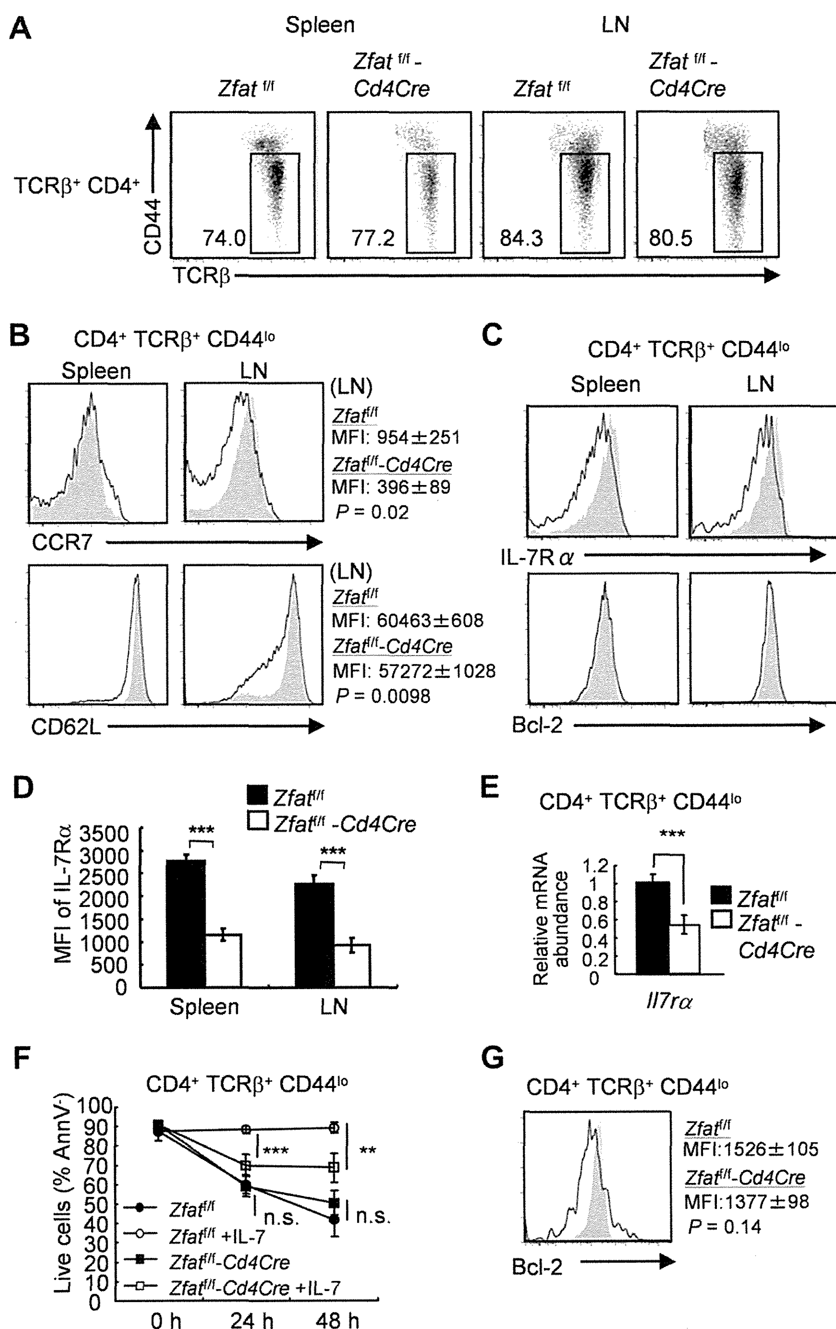

Fig. 3. Reduced expression of IL-7Rα and impaired responses to IL-7 in peripheral CD4$^+$ T cells from the *Zfat*$^{f/f}$*-Cd4Cre* mice. (A) Proportion of naive T cells defined as CD4$^+$TCRβ$^+$CD44^{lo}. Numbers indicate the proportion of the cells within each gate. (B) The expressions of surface CD62L and CCR7 on the splenic and LN CD4$^+$TCRβ$^+$CD44^{lo} T cells from *Zfat*$^{f/f}$*-Cd4Cre* (black line) and *Zfat*$^{f/f}$ (gray-filled) mice. The expressions of surface CD62L and CCR7 on the LN CD4$^+$TCRβ$^+$CD44^{lo} T cells were measured as the mean fluorescence intensity (MFI). The data are the mean ± s.d. of three independent experiments. (C) The expressions of surface IL-7Rα and intracellular Bcl-2 by CD4$^+$TCRβ$^+$CD44^{lo} T cells in the spleen and LN. (D) The expressions of surface IL-7Rα by CD4$^+$TCRβ$^+$CD44^{lo} T cells in the spleen and LN were measured as the mean fluorescence intensity (MFI). The data are the mean ± s.d. of three independent experiments. $^{***}P < 0.01$ (E) Quantitative RT-PCR analysis of IL-7Rα expression by the splenic CD4$^+$TCRβ$^+$CD44^{lo} T cells from *Zfat*$^{f/f}$*-Cd4Cre* (white bars, $n = 9$) and *Zfat*$^{f/f}$ (black bars, $n = 6$) mice. The relative expression for each gene was normalized by expression of *Actb*. The data are the mean ± s.d. of three independent experiments. $^{***}P < 0.01$; n.s., not significant. (F) The proportions of live cells during *in vitro* culture with or without IL-7, and the proportion of live (annexin-V-negative, AnnV$^-$) CD4$^+$TCRβ$^+$CD44^{lo} T cells measured by flow cytometry at the indicated time points. The data are mean ± s.d. of triplicate cultures. $^{**}P < 0.05$; $^{***}P < 0.01$; n.s., not significant ($n = 2$). (G) IL-7-induced Bcl-2 expression by the splenic CD4$^+$TCRβ$^+$CD44^{lo} T cells from *Zfat*$^{f/f}$*-Cd4Cre* (black line) and *Zfat*$^{f/f}$ (gray-filled) mice at 24 h were measured as the mean fluorescence intensity (MFI). The data are the mean ± s.d. of three independent experiments.

CCR7 and CD62L expression were decreased in comparison to those in the *Zfat*$^{f/f}$ mice (Fig. 3B). CD62L expression in the splenic *Zfat*$^{f/f}$*-Cd4Cre* T cells was slightly decreased in comparison to that in the *Zfat*$^{f/f}$ mice (Fig. 3B). These results suggest that the reduced expression of homing receptors would partially account for the reduced peripheral T cell number in the *Zfat*$^{f/f}$ mice. However, considering the fact that reduced numbers of CD4$^+$ T cells were observed throughout the periphery, i.e., in the LN, spleen and peripheral blood (Fig. 1A), other factors rather than homing receptors seem to cause the altered T cell homeostasis in the *Zfat*$^{f/f}$ mice.

Because IL-7Rα- and TCR-mediated signals play a critical role in the survival of naive T cells [16,17], we next examined the IL-7Rα-related profiles in the peripheral CD4$^+$ T cells. IL-7Rα surface expression in the splenic and LN CD4$^+$ T cells, but not in the CD4SP

thymocytes, was significantly decreased in *Zfat*$^{f/f}$-*Cd4Cre* mice in comparison to that in *Zfat*$^{f/f}$ mice (Fig. 2B, Fig. 3C and D), but no difference in the expression of anti-apoptotic protein Bcl-2, a survival factor for peripheral T cells, was observed (Fig. 3C). Consistent with this result, the expression level of *Il7rα* mRNA in naive $CD4^+$ T cells from *Zfat*$^{f/f}$-*Cd4Cre* mouse spleen showed a significant decrease in comparison to that from *Zfat*$^{f/f}$ mouse spleen (Fig. 3E). The proportion of live cells among the $CD44^{lo}CD4^+$ T cells during *in vitro* culture without IL-7 was not significantly different between the genotypes (Fig. 3F), whereas the proportion of live *Zfat*$^{f/f}$-*Cd4Cre* $CD44^{lo}CD4^+$ T cells during the culture with IL-7 was significantly decreased in comparison with that from *Zfat*$^{f/f}$ mice (Fig. 3F). Bcl-2 expression is known to be induced by an IL-7R-mediated signal [30]. Interestingly, the IL-7-induced Bcl-2 expression in naive $CD4^+$ T cells from *Zfat*$^{f/f}$-*Cd4Cre* mice tended to be lower than that in *Zfat*$^{f/f}$ mice, although the difference was not statistically significant (Fig. 3G). These results collectively suggested that Zfat plays a critical role in the proper expression of IL-7Rα and also that the IL-7/IL-7R-mediated signal is impaired, at least in part of Bcl-2 induction, in the $CD44^{lo}CD4^+$ T cells from *Zfat*$^{f/f}$-*Cd4Cre* mice, resulting in a reduction in the number of $CD4^+$ T cells. As transcriptional factors including Foxo1 [26,30,31], Gabpα and Gfi-1 [32], are known to be regulators for the IL-7Rα expression in T cells, the relation between Zfat and these factors should be addressed in the future.

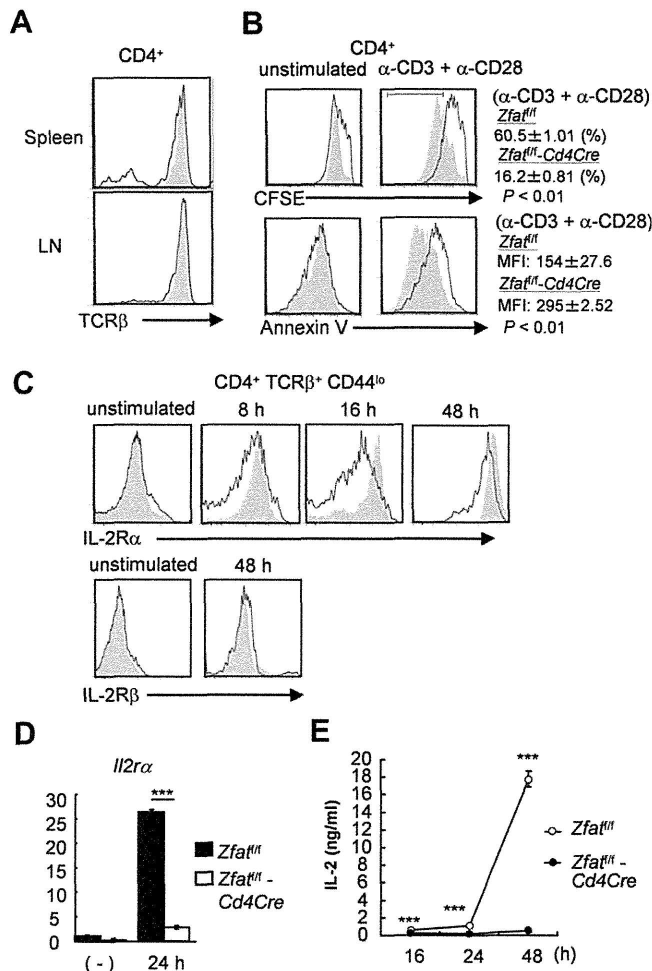

Fig. 4. Impaired proliferation and increased apoptosis with a reduced expression of IL-2Rα and IL-2 upon TCR-stimulation in $CD4^+$ T cells from *Zfat*$^{f/f}$-*Cd4Cre* mice. (A) Surface expression of TCRβ in the splenic and lymph node (LN) $CD4^+$ T cells from *Zfat*$^{f/f}$-*Cd4Cre* (black line) and *Zfat*$^{f/f}$ (gray-filled) mice at 8–12 weeks of age. (B) Cell division analysis using CFSE staining (upper) and apoptotic analysis using annexin-V staining (lower) in the splenic $CD4^+$ T cells from 8- to 12-week-old *Zfat*$^{f/f}$-*Cd4Cre* (black line) and *Zfat*$^{f/f}$ (gray-filled) at 48 h after stimulation with anti-CD3/CD28 antibodies. Data are representative of three independent experiments. Percentages within a panel indicate the fraction of cells that underwent proliferation. The expressions of annexin-V were measured as the mean fluorescence intensity (MFI). The data are the mean ± s.d. of three independent experiments. (C) The surface expression of IL-2Rα and IL-2Rβ on the splenic $CD4^+TCR\beta^+CD44^{lo}$ T cells from *Zfat*$^{f/f}$-*Cd4Cre* (black line) and *Zfat*$^{f/f}$ (gray-filled) mice. The cells were cultured with or without anti-CD3/CD28 antibodies for 8, 16 and 48 h. The data are representative of three independent experiments. (D) Quantitative RT-PCR analysis of the *Il2rα* expression of $CD4^+$ T cells from the indicated mouse spleen before or 24 h after stimulation with anti-CD3/CD28 antibodies. The relative expression for each gene was normalized by expression of *Actb*. One representative data of three independent experiments performed in triplicate. The data are the mean ± s.d. $^{***}P < 0.01$. (E) IL-2 production by stimulation with anti-CD3/CD28 antibodies. The splenic $CD4^+$ T cells from the indicated mice were cultured with plate-coated anti-CD3 and anti-CD28 antibodies. At the indicated times, supernatants were harvested from the cultures and analyzed for IL-2 content using an ELISA. The data are the mean ± s.d. of three independent experiments. $^{***}P < 0.01$.

3.4. Impaired proliferation and increased apoptosis with the reduced expression of IL-2Rα and IL-2 in response to TCR-stimulation in *Zfat*$^{f/f}$-*Cd4Cre* T cells

We next examined the TCR-mediated response in the peripheral $CD4^+$ T cells. The expression of TCRβ in the splenic and LN $CD4^+$ T cells, and the TCR-stimulation-induced ERK activation in the splenic $CD4^+$ T cells were comparable between the genotypes (Fig. 4A, Supplementary Fig. 2B), suggesting that the general TCR-mediated signals were not impaired in *Zfat*$^{f/f}$-*Cd4Cre* $CD4^+$ T cells. To address the functional role of Zfat in T cell response induced by TCR-stimulation, cell division and apoptosis assays were carried out, revealing a decrease in the cell division and an increase in the apoptosis in *Zfat*$^{f/f}$-*Cd4Cre* T cells in comparison to those from *Zfat*$^{f/f}$ mice (Fig. 4B, Supplementary Fig. 3A). Since the IL-2/IL-2R signal plays a critical role in peripheral T cell responses following TCR-stimulation [22], we addressed the expressions of IL-2Rα and IL-2 in *Zfat*$^{f/f}$-*Cd4Cre* T cells. The TCR-stimulation-induced IL-2Rα expression in the splenic T cells, but not the IL-2Rβ expression, was clearly decreased in the *Zfat*$^{f/f}$-*Cd4Cre* mice in comparison to that in *Zfat*$^{f/f}$ mice at 8, 16 and 48 h of TCR-stimulation (Fig. 4C, Supplementary Fig. 3B). The induction of *Il2rα* mRNA expression at 24 h of the stimulation was severely impaired in *Zfat*$^{f/f}$-*Cd4Cre* mice (Fig. 4D), and together these findings suggested that the reduced expression of IL-2Rα in the *Zfat*$^{f/f}$-*Cd4Cre* T cells was, in part, dependent on the reduced expression level of *Il2rα* mRNA. Furthermore, the production of IL-2 after the TCR-stimulation was severely impaired in the *Zfat*$^{f/f}$-*Cd4Cre* T cells (Fig. 4E). These results suggested that the impaired expressions of both IL-2Rα and IL-2 after the TCR-stimulation would account for the reduction in cell division in the *Zfat*$^{f/f}$-*Cd4Cre* T cells.

In this study, we established T cell-specific *Zfat*-deficient mice using *Cd4-Cre* mice and found that Zfat plays crucial roles as a novel cell-intrinsic factor both in peripheral T cell homeostasis and in TCR-mediated cellular response. As both the impaired IL-7Rα- and TCR-mediated signals are likely to underlie the altered T cell homeostasis and T cell survival in *Zfat*$^{f/f}$-*Cd4Cre* mice, elucidation of Zfat functions in the immune system will provide insights into the molecular mechanisms of immune-related diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.065.

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Immune aspects of the pathogenesis of inflammatory bowel disease

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ABSTRACT

Although the precise etiologies of inflammatory bowel disease (IBD) (ulcerative colitis and Crohn's disease) remain obscure, several reports have indicated that dysfunction of the mucosal immune system plays an important role in its pathogenesis. Recent progress with genome-wide association studies has identified many IBD susceptibility genes. In individuals with genetic risk, abnormal interactions between the host immune system and gut flora, and dysregulation of cellular responses such as autophagy and ER stress, induce an abnormal host immune response in the gut resulting in intestinal inflammation. Research progress animal models in IBD, and in human IBD, has identified several key molecules in IBD pathogenesis such as TNFα and adhesion molecules, and molecular targeting therapies based on these molecules have been developed. Here, we review immunological aspects in IBD pathogenesis and the development of immunoregulatory therapy.

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1. Introduction

Inflammatory bowel disease (IBD) is classified according to two typical phenotypes, namely ulcerative colitis and Crohn's disease. Although the precise etiology of IBD remains obscure, several reports have indicated that dysfunction of the mucosal immune system plays an important role in its pathogenesis (Xavier & Podolsky, 2007). The gastrointestinal tract is continuously exposed to a variety of antigens including enteric bacteria and foods. However, homeostasis of the gut is maintained in the normal state, i.e. without the development of intestinal inflammation, by suppressing excessive immune responses to foreign antigens. In both innate and acquired immunity, the disruption of regulatory mechanisms may lead to abnormal immune responses to enteric antigens and cause chronic intestinal inflammation. Here, we review the immunological aspects of the pathogenesis of IBD.

2. Discovery of susceptibility genes indicates immunological dysregulation in IBD

It has been well established that host genetic susceptibility plays a key role in the risk of development of IBD. For example, the risk of development of IBD in homozygous twins is higher than the risk in the general population. In 2001, nucleotide oligomerization domain receptor (NOD) 2, also known as CARD15, an intracellular pathogen recognition molecule, was identified as a susceptibility gene for Crohn's disease by linkage analysis (Hugot et al., 2001; Ogura et al., 2001). Since the discovery of NOD2, innate immune response has been highlighted in the research of IBD pathogenesis. More recently, genome-wide association studies (GWAS) have enabled the identification of approximately 99 single nucleotide polymorphisms that confer risk for either ulcerative colitis or Crohn's disease. The observation that several risk loci are shared between ulcerative

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colitis and Crohn's disease (Franke et al., 2010; Anderson et al., 2011) suggests that there is some overlap of disease pathogenesis. Importantly, many of these recently identified IBD susceptibility genes are associated with host immune function including epithelial barrier function, host defense mechanisms in response to pathogens, and host immune responses (Fig. 1). For example, *NOD2* (Hugot et al., 2001; Ogura et al., 2001), *CARD9* (Zhernakova et al., 2008)and *ITLN1* (Barrett et al., 2008) in the innate immune response; *IL-23R* (Barrett et al., 2008), *STAT3* (Barrett et al., 2008), and *TNFSF15* (Yamazaki et al., 2005) in the IL-23-Th17 pathway; *ATG16L1* (Hampe et al., 2007; Prescott et al., 2007) and *IRGM* (Parkes et al., 2007) in autophagy; *XBP-1* (Kaser et al., 2008) and *ORMDL3* (McGovern et al., 2010) in ER stress; and protein–tyrosine phosphatase, non-receptor type*(PTPN)2* (Wellcome Trust Case Control Consortium, 2007; Wiede et al., 2011) in T cell response, have been identified. Research into IBD susceptibility genes has also shown differences in genetic risk between several human races. In Japanese IBD patients, many of the IBD susceptibility genes identified in other populations (including *NOD2*, *IL-23R*, and *ATG16L1*) did not show any association (Inoue et al., 2002; Yamazaki et al., 2007). *TNFSF15*, originally identified as a Crohn's disease susceptibility gene in a Japanese population, showed a lesser association in one European population (Picornell et al., 2007; Tremelling et al., 2008), but it showed a definite association in another European cohort (Thiebaut et al., 2009).

Thus, many susceptibility genes and loci have been identified by GWAS; however, the functional roles of these genetic loci are not fully understood. Glocker et al. identified children with mutations in the interleukin-10 receptor (*IL-10R*) (Glocker et al., 2009) and *IL-10*genes (Glocker et al., 2010). Kotlarz et al. (2012) identified loss of function mutations in *IL-10* and *IL-10R* in patients with very early onset IBD. These findings indicate that infant IBD patients with perianal disease should be screened forIL-10 and IL-10R deficiency.

3. Cellular responses — autophagy and ER stress

Recent advances in GWAS have identified several IBD susceptibility genes which are associated with cellular responses such as autophagy and endoplasmic reticulum (ER) stress. These cellular responses play a homeostatic role in epithelial barrier function and host immune responses.

3.1. Autophagy

Autophagy-related gene 16-like1 (*ATG16L1*) and *IRGM*are two genes recently identified by GWAS as Crohn's disease susceptibility genes, both of which are associated with "autophagy" (Hampe et al., 2007; Parkes et al., 2007; Prescott et al., 2007; Rioux et al., 2007). Autophagy is one mechanism for maintaining cellular homeostasis, and means "to eat oneself" or "self-cannibalization." While apoptosis is a cell death pathway, autophagy is involved in recycling cellular organelles for cell survival. Autophagy is now also considered to be important for host defense against intracellular microorganisms. The *ATG16L1* gene is located in chromosome 2q37.1 and encodes a protein that is known to mediate resistance against intracellular microorganisms, such as bacteria, and viral particles. Interestingly, *ATG16L1* did not show a positive association with Crohn's disease in a Japanese population, similar to the case for *NOD2/CARD15* studies (Inoue et al., 2002; Yamazaki et al., 2007). The association of these autophagy genes with Crohn's disease strongly supports the hypothesis that abnormal function in elimination and innate immune responses to intracellular pathogens contributes to the pathogenesis of Crohn's disease. In contrast to a report by Rioux et al. (Hampe et al., 2007; Parkes et al., 2007; Prescott et al., 2007; Rioux et al., 2007), Fujita et al. reported that in *Atg16L1*-deficient mouse embryonic fibroblasts with a stably expressed WD repeat domain mutant of *Atg16L1*, an *Atg16L1* WD repeat domain deletion and the T300A mutant have little impact on *Salmonella* infection (Fujita et al., 2009).

Another possible pathogenic contribution of a mutation of *ATG16L1* is associated with Paneth cell function and differentiation. Cadwell K et al. demonstrated that Paneth cells of *Atg16L1*-deficient (*Atg16L1*^{HM}) mice exhibit notable abnormalities in the granule exocytosis pathway. Importantly, Crohn's disease patients homozygous for the *ATG16L1* Crohn's disease risk allele displayed Paneth cell granule abnormalities similar to those seen in this mouse model (Cadwell et al., 2008). An

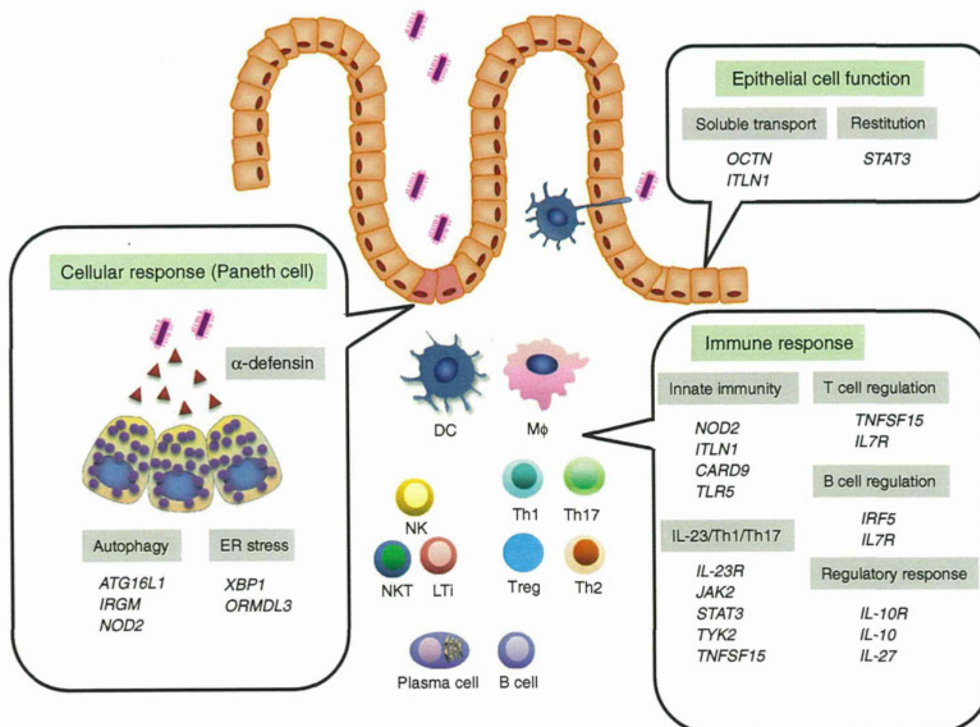

Fig. 1. Susceptibility genes in the immunopathogenesis of IBD. Recently identified IBD susceptibility genes suggest disruption of immunological regulation in IBD pathogenesis including epithelial barrier function, immune response (e.g. innate immunity, IL-23/Th17 pathway), and cellular responses (e.g. autophagy, ER stress).

interaction between a specific strain of an enteric virus, murine norovirus (MNV) and *ATG16L1* mutation has been reported (Cadwell et al., 2010). MNV infection caused morphological and granule packaging abnormalities in Paneth cells of *Atg16L1*^{HM} mice, but not in those of wild type mice. Interestingly, in *Atg16L1*^{HM} mice infected with MNV, DSS treatment exhibited multiple histopathological characteristics of human Crohn's disease including muscular layer inflammation, mesenteric fat and blood vessels, lymphoid aggregates, and subserosal fibrosis.

Analysis using intestinal biopsies from patients with pediatric Crohn's disease showed that autophagy is specifically activated in Paneth cells, that this activation is associated with a significant decrease in the number of secretory granules and with features of crinophagy, and that it occurs independently of *ATG16L1* or *IRGM* variants, which are associated with Crohn's disease (Thachil et al., 2012). Plantinga et al. analyzed the response to NOD2 ligands in peripheral blood mononuclear cells (PBMCs) from healthy individuals and patients with Crohn's disease with different *ATG16L1* genotypes. PBMCs from individuals with the *ATG16L1* Thr300Ala risk variant displayed increased production of the pro-inflammatory cytokines IL-1β and IL-6, after stimulation with NOD2 ligands compared with other genotypes (Plantinga et al., 2011). Collectively, ATG16L1 plays several important roles in autophagy, innate host defense by Paneth cells, and regulation of proinflammatory response (Fig. 2). Mutation of *ATG16L1* may lead to abnormal cellular responses in Crohn's disease.

3.2. ER stress

The unfolded protein response (UPR) has been identified as a critical pathway in the maintenance of cellular homeostasis. *De novo* protein synthesis occurs in the ER, and the folded proteins are then transported to Golgi apparatus. However, in the case of unfolded or misfolded proteins, these are stored in the ER. Excess accumulation of these structurally abnormal proteins will cause ER stress, and finally induces apoptotic cell death and may cause several inflammatory and degenerative diseases. The UPR is important in a variety of cell phenotypes, especially in secretory cells such as goblet cells and Paneth cells in the intestine. The UPR is initiated by binding of unfolded proteins with glucose-regulated protein 78 (GRP78) in the ER. Subsequent recognition of these unfolded proteins leads to homeostatic cellular responses such as ER-associated degradation and translational responses through the pancreatic endoplasmic reticulum kinase (PERK)-elongation initiation factor 2α (eIF2α) pathway and the inositol-requiring enzyme 1(IRE-1)-X-box binding protein 1 (XBP1) pathway (Walter & Ron, 2011) (Fig. 3).GWAS have identified the association of several ER stress associated genes with IBD susceptibility (Barrett et al., 2008; Kaser et al., 2008; McGovern et al., 2010). An intestinal epithelial cell (IEC)-specific isoform of *Ire1* deficient (*Ire1β*$^{-/-}$) mice showed increased susceptibility to dextran sodium sulfate (DSS)-induced colitis (Bertolotti et al., 2001), suggesting dysregulation of UPR in IECs may become a trigger for chronic intestinal inflammation. Subsequent to the report of *Ire1β*$^{-/-}$ mice, Kaser et al. (2008) demonstrated that mice with an IEC-specific deletion of *Xbp1* (*Xbp1*$^{Vil\text{-}fl/fl}$ mice) developed spontaneous small intestinal inflammation. Furthermore, in *Xbp1*$^{Vil\text{-}fl/fl}$ mice, Paneth cells and goblet cells (which are typical secretory epithelial cell phenotypes) showed numerically, structurally and functionally abnormal development. *Xbp1*$^{Vil\text{-}fl/fl}$ mice exhibited increased susceptibility to *Listeria monocytogenes* infection, as did *Nod2*$^{-/-}$ mice (Kobayashi et al., 2005).

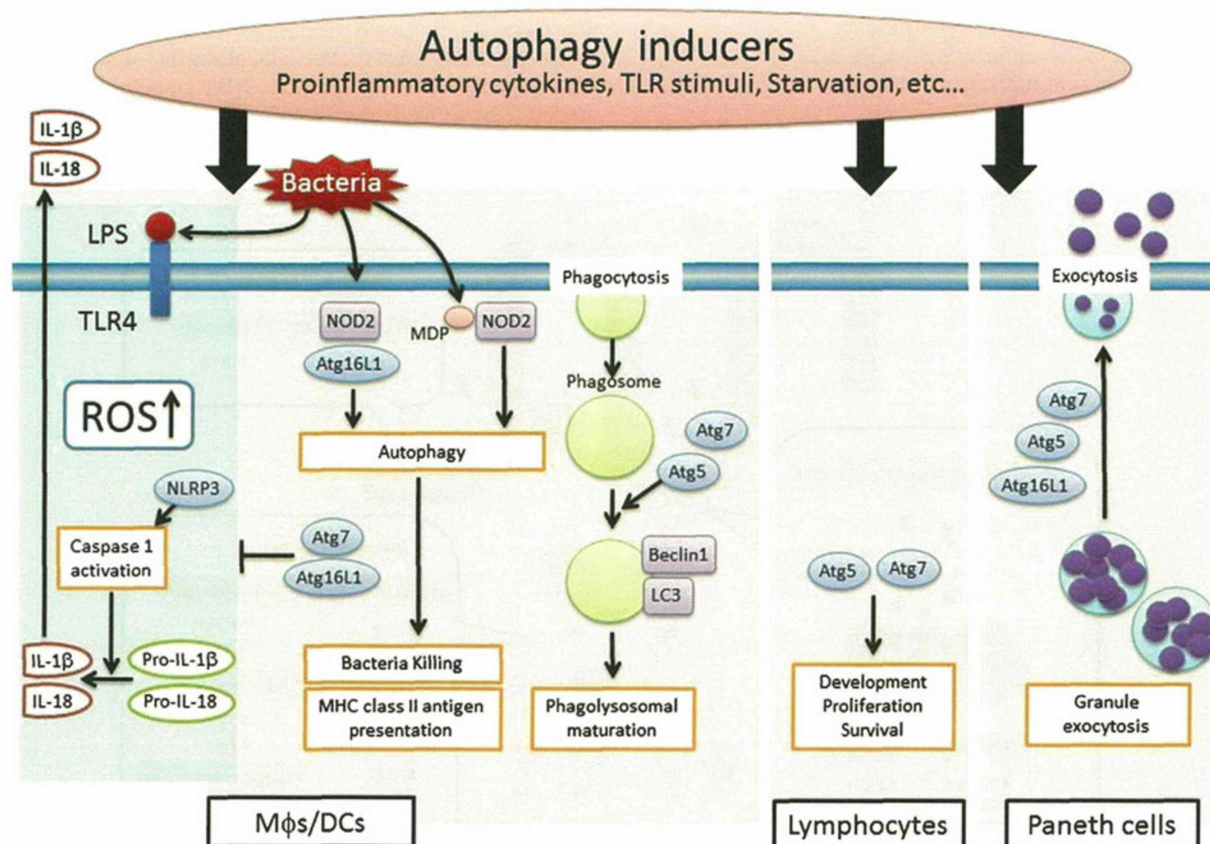

Fig. 2. Contribution of ATG16L1 in cellular responses. Autophagy inducers (e.g. proinflammatory cytokines, TLR stimuli, starvation) induce not only autophagy, but also several cellular responses. LPS/TLR4 signal promotes cleavage of pro-IL-1β and pro IL-18 and induce the production of IL-1β and IL-18 in the macrophages and DCs. In another pathway, NOD2 recognizes bacterial muramyl-dipeptide (MDP) and recruits ATG16L1. Subsequently, autophagy is induced resulting in bactericidal effects and presentation of endogenous antigens to MHC class II. These processes are impaired in patients with mutation in NOD2 or Atg16L1. Dysregulated autophagy caused by the mutation in also affects the excessive production of proinflammatory cytokines, such as IL-1β and IL-18. Autophagy genes, Atg5 and Atg7, promote autophagosomal formation and maturation. Atg5 and Atg7 contribute to the development, proliferation and survival in lymphocytes, and promote granuleexocytosis together in Paneth cells.ATG16L1 is also involved in this process in the Paneth cells.

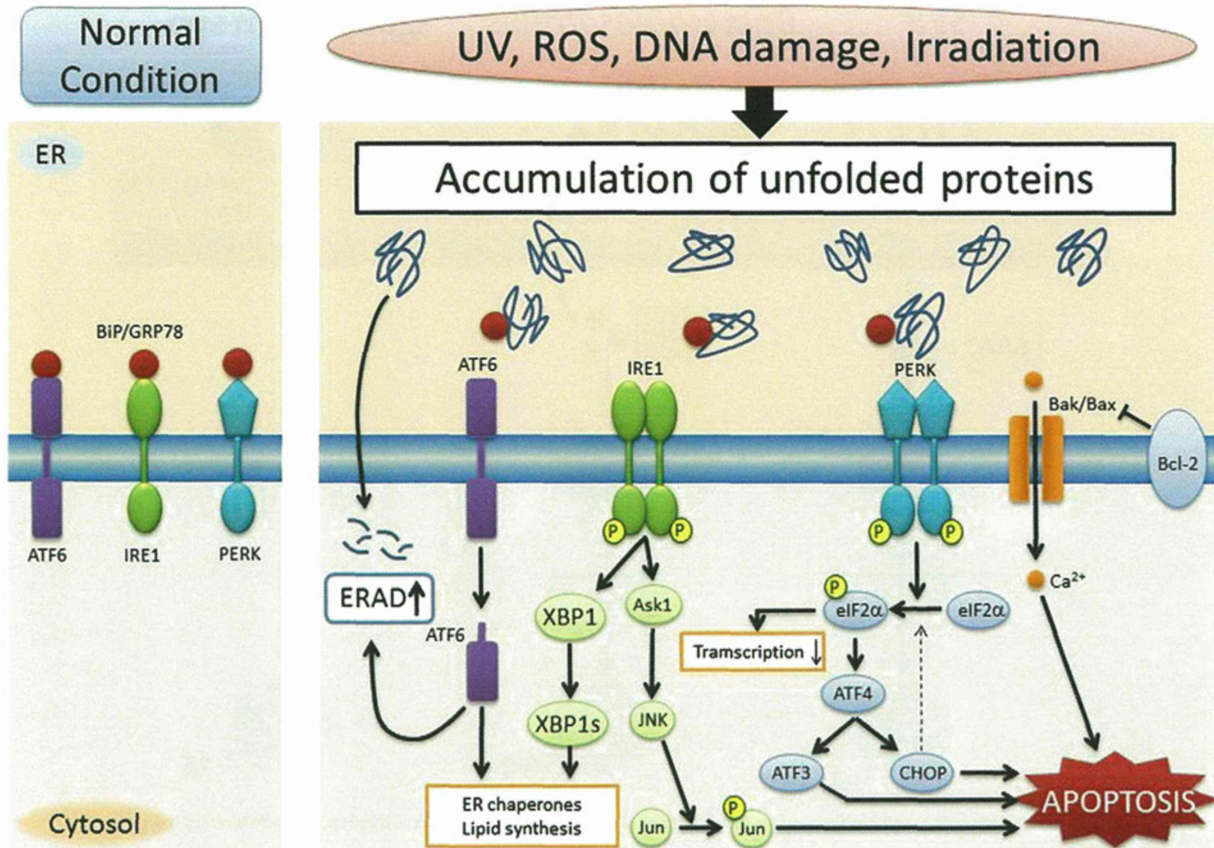

Fig. 3. ER stress induces cellular responses. Under the normal condition, three main ER stress transducers activating transcription factor (ATF) 6, IRE1, and PRKR-like endoplasmic reticulum kinase (PERK) are inactive due to binding to BiP (immunoglobulin heavy-chain binding protein, also referred to as glucose-regulated protein (GRP) 78). When unfolded proteins accumulate in the ER lumen, BiP dissociates from the ER stress transducers. Dissociation of BiP activates them. BiP-free ATF6 translocates to the Golgi apparatus and is cleaved from the membrane. This active form of ATF6 induces transcription of ER chaperones, such as BiP, and XBP1 and promotes export of misfolded proteins and ER-associated protein degradation (ERAD). Active IRE1 has an endoribonuclease activity and causes frame switch splicing of XBP1 mRNA. The spliced form of XBP1 (XBP1s) protein has potential transcription activity and regulates genes involved in ER chaperones. These mechanisms clear misfolded proteins and restore ER homeostasis. On the other hand, IRE1 also activates JNK and stimulate the phosphorylation of Jun. Active PERK phosphorylates eIF2α, which results in successive downregulation of protein translation. eIF2α also induces preferential translation of transcription factor ATF4. Thereby, ATF4 induces expression of C/EBP-homologous protein (CHOP) and ATF3. These proteins trigger an apoptotic program.

Importantly, several key players in IBD pathogenesis (NOD2, ATG16L1, XBP1, and α-defensin) can be found together in Paneth cells, strongly suggesting that Paneth cells play a key role in IBD pathogenesis.

4. Microbiota and IBD

4.1. Host–microbe interactions for gut immunological homeostasis

The mammalian intestine is home to ~100 trillion bacteria that perform important metabolic and protective functions for their hosts. Interactions between the host immune system and the resident microbes have been highlighted as particularly important in this context. In mouse studies, it has become evident that enteric flora regulate intestinal immune cell development. In differentiation of IL-17 producing memory T cells (T helper 17 (Th17) and T regulatory (Treg) cells), a delicate balance of soluble factors, such as transforming growth factor (TGF)-β and IL-6, plays an essential role (Weaver et al., 2006; Stockinger & Veldhoen, 2007). The recent findings that commensal bacteria can regulate the development of both Th17 and Treg cells suggests the importance of the local environment induced by commensal microorganisms in immunological homeostasis of gut-associated lymphoid tissues (GALT) (Fig. 4). Ivanov II. et al. demonstrated that a single commensal small intestinal microbiota, segmented filamentous bacterium (SFB), is sufficient to induce the appearance of Th17 cells in the lamina propria. Interestingly, this exciting discovery that a single commensal microbiota can induce a specific T cell subset is derived from the comparative analysis of the intestinal microbiota in B6 mice bred in Taconic Farms compared with those bred in the Jackson Laboratory. SFB were present in conventionally raised B6 mice from Taconic Farms but were undetectable in the same strain of mice obtained from the Jackson Laboratory. Colonization of germ free mice by SFB induced intestinal Th17 cells. In addition, the introduction of fecal material from germ free mice colonized with SFB into Jackson B6 mice by oral gavage induced robust Th17 cell differentiation. SFB colonization resulted in reduced growth of an intestinal pathogen, suggesting that intestinal commensal microbes can contribute to Th17 cell-mediated mucosal protection (Ivanov et al., 2009). In addition, Atarashiet al. reported that commensal bacteria-derived adenosine 5′-triphosphate (ATP) activates a unique subset of colonic lamina propria cells, $CD70^{high}CD11c^{low}$ dendritic cells (DCs) cells, leading to the differentiation of Th17 cells. A $CD70^{high}CD11c^{low}$DC subset expresses Th17-prone molecules, such as IL-6 and IL-23p19, in response to ATP stimulation, and preferentially induces Th17 differentiation of co-cultured naive CD4+ T cells (Atarashi et al., 2008). These observations highlight the importance of commensal bacteria for Th17 differentiation in health and disease. Not only the development of Th17 cells, but also that of Foxp3transcription factor-positive Treg cells, are regulated by commensal bacteria. Atarashi et al. reported indigenous *Clostridium* species promotecolonic Treg cell accumulation. Colonization of mice by a defined mix of *Clostridium* strains provided an environment rich in TGF-β, and this affected Foxp3+ Treg cell number and function in the colon (Atarashi et al., 2011). *Bacteroides fragilis* may also regulate the promotion of Treg development. Mazmanianet al. reported that the prominent human symbiont *B. fragilis* protects animals from experimental colitis induced by

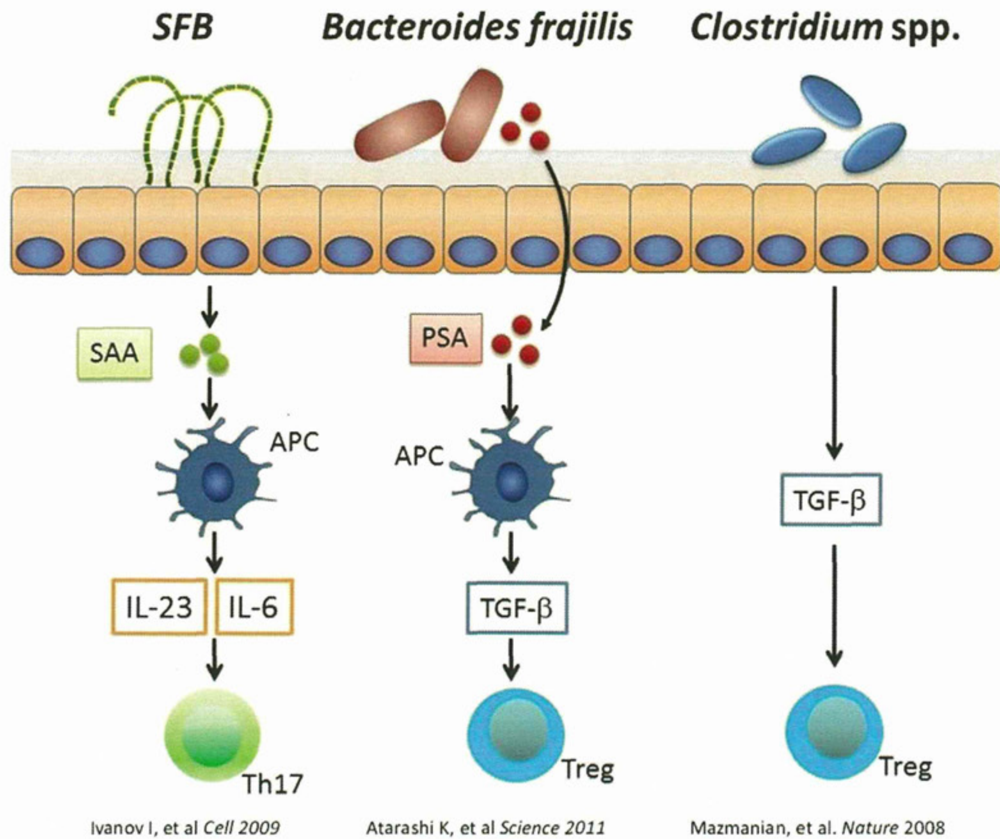

Fig. 4. Intestinal microbiota regulates intestinal mucosal immunology. Enteric flora regulate intestinal immune cell development. SFB induces Th17 cells. *Clostridium* species promoteTreg cell accumulation. PSA derived from *Bacteroides fragilis* also promotes Treg development.PSA; polysaccharide A.

Helicobacter hepaticus. This beneficial activity requires a single microbial molecule, polysaccharide A (PSA). In animals harboring *B. fragilis* not expressing PSA, *H. hepaticus* colonization leads to disease and pro-inflammatory cytokine production in colonic tissues. PSA is required to suppress pro-inflammatory interleukin-17 production by intestinal immune cells and protects from inflammatory disease through an IL-10-producing CD4+Treg cell type (Mazmanian et al., 2008). Further investigations by Roundet al. revealed that PSA derived from *B. fragilis* directs the development of Foxp3$^+$T cells. Monocolonization of germ-free animals with *B. fragilis* increases the suppressive capacity of Tregs and induces anti-inflammatory cytokine production exclusively from Foxp3$^+$ T cells in the gut (Round & Mazmanian, 2010). The homeostatic functional property of PSA from *B. fragilis*is through Toll-like receptor (TLR)2-dependent signal transduction on Foxp3$^+$Tregs (Round et al., 2011). For the development of colonic Tregs, commensal bacteria antigen-specific post-thymic education is important. Colonic Tregs use T-cell antigen receptors (TCRs) different from those used by Tregs in other locations. Many of the local antigens seemed to be derived from commensal bacteria. These TCRs did not facilitate thymic Treg cell development, implying that many colonic Treg cells arise by antigen-specific peripheral education (Barrett et al., 2008).

It also seems likely that host immunological properties might affect the composition and function of the enteric flora. Mice with T-bet deficiency in theinnate immune system (T-bet−/− x RAG2−/− mice) developed spontaneouscolitis. This colitis was observed to be communicable to genetically intact mice, suggesting loss of T-bet influences bacterialpopulations to become colitogenic (Garrett et al., 2007). Deficiency of the NOD-like receptor family pyrin domain containing (NLRP) 6 in mouse colonicepithelial cells resulted in reduced IL-18 levels and altered fecal microbiota, characterized by expanded representation of the bacterial phyla *Bacteroidetes*(*Prevotellaceae*). NLRP6 inflammasome-deficient mice were characterized by spontaneous intestinal hyperplasia, inflammatory cell recruitment, and exacerbation of DSS-induced colitis. Cross-fostering and co-housing experiments revealed that the colitogenic activity of this microbiota is transferable to neonatal or adult wild-type mice. Wild type (WT) mice cohoused with antibiotic treated NLRP6 deficient mice developed significantly less-severe DSS colitis compared to WT mice cohoused with untreated NLRP6 deficient mice. This reduction in severity correlated with decreased abundance of Prevotellaceae. These findings indicated the role of *Prevotellaceae* as a key representative of this microbiota-associated phenotype (Elinav et al., 2011). Anti-microbial peptides produced by intestinal epithelial cells or Paneth cells play an important role in host innate immunity. RegIIIγ, a secreted antibacterial lectin, is essential for maintaining host-bacterial segregation at the small intestinal epithelial surface. In*RegIIIγ*$^{-/-}$ mice, increased bacterial colonization of the intestinal epithelial surface and activation of intestinal immune responses by the microbiota were observed. Thus, RegIIIγ regulates the spatial relationships between microbiota and host (Vaishnava et al., 2011). Paneth cell α-defensins are antimicrobial peptides that contribute to host defense against enteric pathogens, as well as governing intestinal microbial ecology. In analysis of the intestinal microbiota of mice expressing a human α-defensin gene, and in mice lacking an enzyme required for the processing of mouse α-defensins, significant α-defensin-dependent changes in microbiota compositionwere observed. Furthermore, human α-defensin gene-expressing mice had striking losses of segmented filamentous bacteria and fewer Th17 cells (Salzman et al., 2010). Thus, symbiosis of commensal microorganisms contributes to intestinal immunological homeostasis and protection from pathogens, while dysbiosis of commensal bacteria induces abnormal immune responses and causes intestinal inflammation.

However, in contrast to data from mouse studies, evidence suggesting that a single commensal bacteria strain can regulate the host immune system has not yet been reported in humans. In human microbiota studies, there are some methodological difficulties such as the bacterial culturing systems, and differences in the results from culture-independent and culture-based approaches. Recent progress in anaerobic culturing techniques using gnotobiotic animals, and in metagenomic techniques, make it possible to retrieve components of microbiota that have coexisted in single donors who have physiologic or disease phenotypes of interest (Goodman et al., 2011). Systematic analysis of the human gut microbiome has also progressed rapidly. Intensive global research combining fecal metagenomes of individuals from four countries identified three robust clusters or "enterotypes" that are not specific to a single nation or continent. The enterotypes are mostly driven by species composition, butabundant molecular functions are not necessarily provided by abundant species and are often provided by low-abundance species (Arumugam et al., 2011). The National Institutes of Health-funded Human Microbiome Project Consortium has established a population-scale framework to develop metagenomic protocols, resulting in abroad range of quality-controlled resources and data including standardized methods for creating, processing and interpreting distinct types of high-throughput metagenomic data,a nd these have been made available to the scientific community. Results derived from human microbiome analysis might be useful for diagnosis and prediction or prognosis of several human diseases such as IBD.

The incidence of IBD has increased considerably over the past half century, matching developments in cultural Westernization. The number of patients with IBD is also increasing in Asian countries. The rapid increase in the incidence of IBD cannot be explained by genetic drift. It may be caused by exposure to non-genetic factors, such as diet and lifestyle, in genetically susceptible individuals, leading to the abnormal host immune responses that are characteristic of IBD. So far, the hypothesis that an interaction between genetic background, diet, and flora causes chronic intestinal inflammation has not been proved in mice models of colitis, although it is considered a promising hypothesis to explain IBD pathogenesis. Recent findings reported by Devkota et al. have clearly demonstrated that the interaction between flora and diet is important for the development of chronic intestinal inflammation in genetically susceptible mice. Consumption of a diet high in saturated (milk-derived)fat, but not polyunsaturated (safflower oil) fat, changed the conditions for microbial assemblage and promoted the expansion of a sulfite-reducing pathobiont, *Bilophila wadsworthia*, which is associated with a pro-inflammatory Th1 immune response and increased incidence of colitis in IL-10 deficient mice. The milk-derived-fat promoted taurine conjugation of hepaticbile acids, which increased the number of sulfite-reducing microorganisms such as *B. wadsworthia*. Thus, dietary fats, by promoting changes in host bile acid composition, can alter the composition of gut flora, resulting in dysbiosis that can induce chronic intestinal inflammation (Devkota et al., 2012).

Exposure to microbes during early childhood is associated with protection from immune-mediated diseases such as IBD (von Mutius, 2007). Age-sensitive exposure to commensal microbes is critical for establishing mucosal immune tolerance mechanisms. Olszak et al. showed that colonization of neonatal GF mice with a conventional microbiota protected the animals from mucosal invariant natural killer T (NKT) cell accumulation and related intestinal inflammation (Olszak et al., 2012).

4.2. Pathogenic contribution of gut microbiotain human IBD

Adherent *Escherichia coli* have been assigned a putative role in Crohn's disease (Darfeuille-Michaud et al., 1998; Swidsinski et al., 2002; Martin et al., 2004). Boudeauet al. isolated pathogenic adherent-invasive *E. coli* (AIEC) from the ileal mucosa of a patient with Crohn's disease (Boudeau et al., 1999), and several reports support the contribution of AIEC to the pathogenesis of ileal Crohn's disease (Darfeuille-Michaud et al., 2004; Martinez-Medina et al., 2009).

AIEC may target ileal Peyer's patches (PPs). This strain can interact with mouse and human PPs via long polarfimbriae (LPF). The prevalence of AIEC strains harboring the LPF operon was markedly higher in CD patients compared with controls (Chassaing et al., 2011).

Gelatinase (GelE), a metalloprotease (MMP) from *Enterococcus faecalis*, may play a role in the development intestinal inflammation. Stimulation with *E. faecalis* or purified GelE from patients with Crohn's disease and ulcerative colitis revealed proteolytic activity on epithelial barrier function (Steck et al., 2011).

Mammalian gut contains not only bacterial flora, but also a rich fungal community that interacts with the immune system through the innate immune receptor Dectin-1. Mice lacking Dectin-1 exhibited increased susceptibility to chemically-induced colitis, which was the result of altered responses to indigenous fungi. In addition, a polymorphism in the gene for Dectin-1 (CLEC7A) in humans is strongly linked to a severe form of ulcerative colitis (Iliev et al., 2012).

Recent advances in technology for analysis of human microbiota, such as 16S rRNA sequencing, has made it possible to analyze the microbiota in human IBD (Conte et al., 2006; Sokol et al., 2006; Martinez et al., 2008; Takaishi et al., 2008; Andoh et al., 2011; Frank et al., 2011; Kellermayer et al., 2012; Li et al., 2012). Frank et al. reported the results of a culture-independent rRNA sequence analysis of GI tissue samples obtained from CD and UC patients. Comparison of clone libraries of GI tissue samples reveals statistically significant differences between the microbiota of CD and UC patients and those of non-IBD controls (Frank et al., 2007). Papa et al. have attempted to diagnose pediatric IBD non-invasively by assessing differences in the microbiota using 16S rRNA sequencing analysis of fecal samples (Papa et al., 2012). Furthermore, transplantation of fecal microbiota from healthy donors to patients with IBD has also been reported. Systematic review by Anderson et al. evaluated 17 articles of case series/case reports of patients receiving fecal microbiota transplantation for management of their IBD. Whilst the available evidence is limited and weak, they concluded that fecal microbiota transplantation has the potential to be an effective and safe treatment for IBD (Anderson et al., 2012).

5. Abnormal immune regulation in animal IBD models and human IBD

As the intestinal mucosa is continuously exposed to numerous commensal bacteria and food antigens, it is thought that the gut possesses both innate and acquired regulatory immune systems to prevent excessive inflammatory responses against antigen stimulation. As susceptibility gene profiles reveal, disruption of the innate and acquired immune systems of the gut may cause the development of chronic intestinal inflammation. Gut innate and acquired immunity is composed of several key players, such as intestinal epithelial cells (IECs), macrophages (Mϕs), DCs, NKT cells, innate lymphoid cells (ILCs), T cells, and B cells.

5.1. Intestinal epithelial cells

Intestinal epithelial cells play a role as a barrier to prevent invasion by pathogens, and the influx of antigens. IECs produce mucins and trefoil factors which are important protective components of the mucus layer covering the surface of the intestinal lumen. IECs also produce several types of anti-microbial peptide such as defensins, which are a classical innate immune mechanism. Therefore, disruption of this barrier system may allow invasion by not only pathogenic micro-organisms, but also commensal bacteria and food antigens. IECs also act as sensors for pathogens through several innate immune receptors such as toll-like receptors (TLRs) and NODs, and produce cytokines and chemokines to recruit immune cells. TLR signaling pathways induce the production of pro-inflammatory cytokines and chemokines in

IECs, and also affect epithelial integrity. The activation of TLR2 signaling preserves IEC integrity by claudin 2 up-regulation through PI3 kinase and MyD88 pathways (Cario et al., 2007). Claudins 2, 5 and 8, tight junction proteins in IECs, exhibit altered expression levels and distribution in Crohn's disease. Destruction of tight junction structures may cause increased permeability and bacterial translocation (Zeissig et al., 2007). Restitution of IECs is important for rapid recovery of the mucosal barrier. Recently, IL-22, which belongs to the IL-10 family of cytokines, has been reported to enhance STAT3-dependent expression of mucus-associated molecules and restitution of goblet cells (Sugimoto et al., 2008). Recent studies have demonstrated that a major cell type producing IL-22 is ILCs, suggesting that ILCs modulate the barrier function of IECs (Spits & Di Santo, 2011).

IECs produce constitutive and inducible anti-microbial peptides. In particular, Paneth cells located at the bottom of the crypt have intracellular granules that produce α-defensin. Paneth cells expressing TLRs and NOD2 may play a role as pathogen sensors, and thus contribute to maintenance of the crypt environment. Nudinget al. demonstrated reduced mucosal antimicrobial activity in Crohn's disease of the colon (Nuding et al., 2007). Wehkamp et al. reported that antimicrobial activity was decreased in Crohn's disease patients with ileal lesions, with a specific decrease of α-defensin production by Paneth cells being observed (Wehkamp et al., 2005). Recent studies by the same group revealed a link between α-defensin deficiency in ileal Crohn's disease and Wnt/Tcf-4, which is known as a regulator of Paneth cell differentiation (Wehkamp et al., 2005). Tanabe et al. have reported that s–s binding of α-defensin stored in Paneth cells is important for escape from the degradation induced by protease activity. Interestingly, they also reported that some Crohn's disease patients have an abnormally denatured form of α-defensin that lacks s–s binding (Tanabe et al., 2007). As a novel function of defensins, Shi et al. found that MMP-7-deficient mice, which do not produce the mature form of α-defensin, are susceptible to DSS-induced colitis (Shi et al., 2007). Thus, abnormal function of antimicrobial peptides, especially α-defensins produced by Paneth cells, alters intestinal microbial ecology (Salzman et al., 2010)and may play at least a partial role in the pathogenesis of Crohn's disease.

Recently, very important findings regarding the role of cross-talk between IECs and immune cells were reported. NF-κB signaling in IECs plays an important role in gut immune homeostasis (Nenci et al., 2007; Zaph et al., 2007). Nenchiet al. reported that mice lacking NEMO (also named IKKγ) in their IECs conditionally developed spontaneous colitis. In NEMO knockout mice, the number of apoptotic IECs was increased, resulting in deterioration of the integrity of the epithelial barrier. Furthermore, these mice exhibited decreased production of the antimicrobial defensin peptides produced by IECs. These changes led to translocation of enteric flora and recruitment of innate immune cells (Nenci et al., 2007).Günther et al. demonstrated that caspase-8 in IECs plays a key role in protecting these cells from TNF-α-induced necroptotic celldeath. Mice with a conditional deletion of caspase-8 in IECs ($Casp8^{\Delta IEC}$) spontaneously developed inflammation in the terminal ileum and were highly susceptible to colitis. $Casp8^{\Delta IEC}$ mice showed increased TNF-α-induced cell death in the Paneth cell area of small intestinal crypts associated with increased expression of receptor-interacting protein (RIP) 3. They also observed high levels of RIP3 in human Paneth cells and increased necroptosis in the terminal ileum of patients with Crohn's disease (Gunther et al., 2011). Rimoldi et al. reported that thymic stromal lymphopoietin (TSLP) produced by IECs leads DCs toward a suppressive phenotype (IL-10 production, but no IL-12 production) and suppresses excessive Th-1 immune responses and, importantly, that the production of TSLP by IECs may be decreased in patients with Crohn's disease (Rimoldi et al., 2005).

5.2. Macrophage and dendritic cells

Mϕs are the major population of tissue-resident mononuclear phagocytes, and play a key role in bacterial recognition and elimination, as well as in the polarization of innate and adaptive immunity. Besides these classical antibacterial immune roles, it has recently become evident that Mϕs are also important for the maintenance of homeostasis, for example, dampening inflammation via the production of anti-inflammatory cytokines such as IL-10 and TGF-β (Mosser, 2003; Mantovani et al., 2004; Gordon & Taylor, 2005). Recent studies have shown that M1- and M2-Mϕs are functionally polarized in response to microorganisms and host mediators. M1-Mϕs are characterized by the production of pro-inflammatory cytokines such as TNF-α, IL-12, and IL-23, while M2-Mϕs are characterized by an IL-10-producing phenotype (Mantovani et al., 2004). Interestingly, it was previously reported that intestinal Mϕs have immune-regulatory functions. In contrast to splenic macrophages, intestinal Mϕs do not express innate response receptors (Rogler et al., 1998; Smith et al., 2001), and although these cells retain their phagocytic and bactericidal functions, they do not produce pro-inflammatory cytokines in response to several inflammatory stimuli, including microbial components (Kamada et al., 2005; Smythies et al., 2005). Importantly, several independent studies have revealed that murine intestinal Mϕs produce the anti-inflammatory cytokine IL-10 (M2-Mϕ) and contribute to maintaining homeostasis of the intestinal immune system. Hirotaniet al. demonstrated that wild type colonic lamina propria Mϕs (LP-Mϕs) were different from splenic Mϕs, as they produced higher amounts of IL-10 in response to pathogen-associated molecular patterns (Hirotani et al., 2005). Kamada et al. also demonstrated in vitro that Mϕs differentiated by macrophage colony-stimulating factor (M-CSF) and intestinal $CD11b^{+}$ Mϕs produced abundant IL-10 in response to whole bacteria stimulation (Kamada et al., 2005). M-CSF is a key growth factor for the development of intestinal Mϕs, as shown by the number of intestinal Mϕs in M-CSF-deficient *op/op* mice being significantly decreased (Cecchini et al., 1994). These results suggest that intestinal Mϕs in wild type mice are the IL-10 producing M2 type and may contribute to the maintenance of homeostasis. Uniquely, Takada et al. analyzed $CD11b^{+}$LP-Mϕs from mice and demonstrated they could be divided into two sub-populations (LP-Mϕ1 and LP-Mϕ2) by flow cytometry analysis (Takada et al., 2010). LP-Mϕ2 expressed CCR2 and produced large amounts of IL-10. Interestingly, $Mcp1^{-/-}$ mice contained fewer LP-Mϕ2 cells, resulting in the exacerbation of DSS-induced colitis, suggesting that LP-Mϕs contribute to the maintenance of gut immune homeostasis by producing IL-10. LP-Mϕs may have antigen presenting functions and induce the differentiation of $FoxP3^{+}$ Tregs that are dependent on IL-10 and retinoic acid (Denning et al., 2007). Thus, recent studies have suggested that Mϕs located in the intestinal mucosa play an important role in the maintenance of intestinal homeostasis by protecting the host from foreign pathogens and negatively regulating excess immune responses to commensal bacteria (Schenk & Mueller, 2007).

Because of this important role of Mϕs in the intestinal mucosa, immune homeostasis in the gut is disrupted when intestinal Mϕ function is dysregulated, and this may result in chronic intestinal inflammation. $IL\text{-}10^{-/-}$ mice develop spontaneous chronic colitis and are widely used as an animal model of human IBD (Kuhn et al., 1993). $IL\text{-}10^{-/-}$ mice develop Th1 polarized immunity in response to the intestinal microbiota, as shown by the observation that $IL\text{-}10^{-/-}$ mice do not develop intestinal inflammation in germ-free conditions (Sellon et al., 1998). This suggests that enteric bacteria play an essential role in the onset and development of colitis in $IL\text{-}10^{-/-}$ mice, which may also be the case in human IBD. Recent studies have demonstrated that antigen presenting cells (APCs), such as Mϕs and DCs, from $IL\text{-}10^{-/-}$ mice are potent activators of Th1 responses (Igietseme et al., 2000) and, importantly, depletion of Mϕs prevented chronic colitis in $IL\text{-}10^{-/-}$ mice (Watanabe et al., 2003). These data suggest that Mϕs and DCs play a key role in the pathogenesis of colitis in $IL\text{-}10^{-/-}$ mice. Kamada et al. demonstrated in vivo that LP-Mϕs from $IL\text{-}10^{-/-}$ mice showed a paradoxical overproduction of IL-12p70 upon bacterial stimuli and that endogenous IL-10 is required for inhibition of IL-12p70 production and