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# Fas Ligand Induces Cell-Autonomous IL-23 Production in Dendritic Cells, a Mechanism for Fas Ligand-Induced IL-17 Production[1]

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**Fas ligand (FasL) has the potential to induce inflammation accompanied by massive neutrophil infiltration. We previously reported that FasL rapidly induces the production of various inflammatory cytokines including IL-1$\beta$ and IL-17. In this study, we investigated the mechanism of the FasL-induced IL-17 production. We found that the culture supernatant of mouse resident peritoneal exudate cells (PEC) cocultured with FasL-expressing tumor (FFL) cells induced IL-17 production in freshly isolated resident PEC. Anti-IL-1$\beta$ Ab strongly inhibited the IL-17-inducing activity. However, rIL-1$\beta$ by itself induced only weak IL-17 production. Intriguingly, anti-IL-12 Ab but not an IL-15-neutralizing agent, IL15R-Fc, strongly inhibited the FasL-induced IL-17-inducing activity. IL-23, which shares the p40 subunit with IL-12, but not IL-12 itself, induced IL-17 production synergistically with IL-1$\beta$ in resident PEC. FasL induced the production of IL-23 in PEC in vivo and in vitro, and IL-17 production following the i.p. injection of FFL cells was severely impaired in p40$^{-/-}$ mice, indicating that IL-23 plays an important role in the FasL-induced IL-17 production. FFL also induced the production of IL-23 in bone marrow- or PEC-derived dendritic cells (DCs). Finally, FasL induced only weak p40 production in a mixture of p40$^{-/-}$ and Fas$^{-/-}$ DC, indicating that FasL induces IL-23 production in DC mainly in a cell-autonomous manner.** *The Journal of Immunology,* **2005, 175: 8024–8031.**

Fas ligand (FasL)[4] expression has been believed to confer immune privilege on the eyes and testes (1, 2). It has been also reported that FasL expression in tumors is a mechanism of tumor immune escape (3). In contrast, the ectopic expression of FasL in vivo induces inflammation accompanied by massive neutrophil infiltration (4–7). The proinflammatory activity of FasL seems to play a pathogenic role, as revealed by using animal models of several inflammatory diseases (8–12). The reason that FasL possesses these contradictory activities, i.e., anti- and proinflammatory activities, has not been well explained. To elucidate this question, we have been investigating the molecular mechanisms of FasL-induced inflammation. First, we discovered that FasL induces the production of mature IL-1$\beta$ in neutrophils by activating caspases, and thus IL-1$\beta$ plays an important role in the FasL-induced inflammation (7). A similar mechanism has been reported to operate on IL-18 production in a murine model of LPS-induced hepatitis (13). Recently, others and we have found that FasL also induces the production, in mice, of other cytokines including IL-6 and IL-17, as well as chemokines including KC, MIP-1, and MIP-2 (14, 15). Unlike IL-1$\beta$ and IL-18, these cytokines do not require caspases for their maturation and secretion. Thus, how FasL induces these cytokines has not been clearly understood.

FasL-induced neutrophil infiltration is markedly, but not completely, diminished in IL-1-deficient mice (7). Therefore, we became interested in the IL-1$\beta$-independent mechanism of FasL-induced neutrophil infiltration. We recently found that FasL induces IL-17 production in peritoneal CD4$^-$CD8$^-$ TCR$\alpha\beta$ or $\gamma\delta$ T cells and plays an important role in the in vivo production of neutrophilic chemokine KC and neutrophil infiltration induced by FasL in IL-1-deficient mice (14). Although FasL-induced IL-17 production is greatly diminished in IL-1-deficient mice, we successfully demonstrated that a small amount of IL-17, sufficient to induce neutrophil infiltration, is produced in vivo in the absence of IL-1. Therefore, we concluded that IL-17 is involved in the IL-1$\beta$-independent FasL-induced inflammation.

IL-23 is a recently identified disulfide-bonded heterodimeric cytokine composed of an IL-23-specific p19 subunit and a p40 subunit common to IL-12 and IL-23, and it is produced by dendritic cells (DCs) and macrophages (16). The IL-23 receptor also consists of two components: an IL-23 receptor-specific subunit and a subunit shared by the IL-12 and IL-23 receptors (17). The biological functions of IL-12 and IL-23 are similar but not identical. IL-23 is not as potent an inducer of IFN-$\gamma$ production as IL-12 (16). Instead, IL-23, but not IL-12, induces the proliferation of memory CD4$^+$ T cells (16) and the production of an inflammatory cytokine, IL-17, in CD4$^+$ T cells (18, 19). Consistently, IL-23 transgenic mice develop multiorgan inflammation associated with neutrophilia (20). Furthermore, mice deficient in IL-23 but not IL-12 are highly resistant to experimental autoimmune encephalomyelitis, whereas mice deficient in IL-12 but not IL-23 are defective in the development of Th1-type immune responses (21).

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[4] Abbreviations used in this paper: FasL, Fas ligand; DC, dendritic cell; PEC, peritoneal exudate cell; BM, bone marrow; PLF, peritoneal lavage fluid, FFL-sup, culture supernatant from resident PECs that were cocultured with FFL; FBH-sup, culture supernatant from resident PECs that were cocultured with FBH.



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These facts suggest that IL-23 has a stronger connection with inflammation and immune pathogenesis than IL-12.

Given these results, in this study, we investigated the mechanism of FasL-induced IL-17 production, and found that FasL induces IL-23 production in DCs, and IL-23 induces IL-17 production in synergy with IL-1$\beta$.

## Materials and Methods

### *Cytokines and neutralizing reagents*

Anti-human FasL mAb (4H9) was described previously. The recombinant mouse IL-12, human IL-15, human IL-23, mouse GM-CSF, IL15R-Fc fusion protein, and anti-mouse IL-12 polyclonal Ab were purchased from R&D Systems. The recombinant mouse IL-1$\beta$ and anti-mouse IL-1$\beta$ polyclonal Ab were purchased from PeproTech and Genzyme, respectively.

### *Mice*

Eight-week-old C57BL/6 and BALB/c mice were purchased from SLC and used between 8 and 12 wk of age. The IL-1-deficient (both the IL-1$\alpha$ and $\beta$ genes were homozygously disrupted) and Fas-deficient C57BL/6 mice were described previously (22). The IL-12 p40-deficient BALB/c mice (C.129S1-Il12btm1Jm/J) were obtained from The Jackson Laboratory. The Kanazawa University Committee on Animal Welfare approved all animal protocols.

### *Cell lines*

The FFL FasL-expressing cell line and the FBH control cell line were described previously (23).

### *Isolation of peritoneal exudate cells (PECs) and peritoneal lavage fluid (PLF)*

The peritoneal cavities of the mice were washed with 1.5 ml of 2% PBS. The peritoneal wash was separated into the cell fraction (PECs) and the supernatant (PLF) by centrifugation at 275 $\times$ *g* for 5 min. The PLF was then passed through a membrane filter (pore size, 0.22 $\mu$m). Resident PECs were prepared from untreated mice. To prepare 4-h and 4-day thioglycolate-PECs, mice received an injection of 1.5 ml of 3% thioglycolate medium (Difco Laboratories) and PECs were prepared 4 h and 4 days after the injection. PECs and PLF from mice who received injections of FFL or FBH cells (FFL- or FBH-PEC and FFL- or FBH-PLF) were prepared as described previously (14). When necessary, FFL and FBH cells were treated with 50 $\mu$g/ml mitomycin C (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% $CO_2$ for 45 min and then extensively washed with HBSS before the injection.

### *Preparation of culture supernatants from resident PECs cocultured with FFL or FBH cells (FFL- or FBH-sup)*

Resident PECs ($5 \times 10^5$ cells/well) were cultured with FFL or FBH cells ($1 \times 10^5$ cells/well) in a 24-well plate. Twenty hours later, the culture supernatants were harvested and passed through a membrane filter (pore size, 0.22 $\mu$m).

### *Cytokine ELISA*

The concentrations of IL-17 and IL-12 p40 in the PLF or culture supernatants were determined using the AN′ALYZA ELISA kit (Genzyme) or OptEIA Set (BD Biosciences) according to the manufacturers' protocols. The concentrations of IL-23 were determined using Ready-SET-Go Mouse IL-23(p19/40) ELISA (eBioscience).

### *RT-PCR analysis for IL-23 p19 mRNA*

RT-PCR analysis was performed as described previously (24). The following primers were used: p19 sense, 5′-gggaacaagatgctggattg-3′; p19 antisense, 5′-atggtagccatgggaacctg-3′; p40 sense, 5′-tgttgtagaggtggactggac-3′; p40 antisense, 5′-ccttcttgtggagcagcaga-3′; p35 sense, 5′-gccacccttgccctcctaaa-3′; and p35 antisense, 5′-caccagcatgcccttgtcta-3′. The amount of template cDNA was adjusted so that a similar amount of a PCR fragment of $\beta$-actin was generated within the linear range of the PCR.

### *DC culture and flow cytometry analysis*

Bone marrow (BM) cells ($2 \times 10^6$ cells) or PECs ($3 \times 10^6$ cells) were cultured in RPMI 1640 medium supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM 2-ME, 20 mM HEPES, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 10 ng/ml GM-CSF (R&D Systems). To determine the proportion of $CD11c^+I\text{-}A^-$ and $CD11c^+I\text{-}A^-$ cells, the cells were pretreated with 5 $\mu$g/ml anti-CD16/CD32 mAb (Fc$\gamma$R blocker; BD Pharmingen) and then stained with FITC-conjugated anti-CD11c mAb (BD Pharmingen) and biotin-conjugated anti-I-Ab mAb (BD Pharmingen) followed by allophycocyanin-conjugated streptavidin (BD Pharmingen). Cells were then analyzed using a FACSCalibur (BD Biosciences).

## Results

### *Resident PEC produce IL-17 production-inducing factor(s) upon FasL stimulation in vitro*

We previously found that the i.p. transplantation of a FasL-expressing cell line (FFL) but not a control cell line (FBH) induces IL-17 production mainly from $CD4^-CD8^-$ TCR$\alpha\beta$ or $\gamma\delta$ T cells in the peritoneal cavity (14). To investigate the mechanism of the FasL-induced IL-17 production, we tested whether FasL induces IL-17 production in resident PECs. As expected, a significant amount of IL-17 was detected in the culture supernatant when resident PECs were cultured with FFL but not FBH cells (Fig. 1*A*). The in vitro IL-17 production induced by FFL was inhibited by an anti-FasL mAb, confirming that FasL is responsible for this response. Next, we tested whether the IL-17-inducing activity was present in the culture supernatant of resident PECs cocultured with FBH or FFL. To this end, we cultured freshly isolated resident PECs in the presence of the culture supernatant from resident PECs that were cocultured with FFL (FFL-sup) or FBH (FBH-sup). Intriguingly, the FFL-sup but not the FBH-sup induced strong IL-17 production in resident PEC (Fig. 1*B*). FFL cells shed soluble FasL as a result of proteolytic cleavage of the membrane-bound FasL. However, a neutralizing anti-FasL mAb did not inhibit the IL-17 production-inducing activity in FFL-sup (Fig. 1*C*). Consistent with this, purified soluble FasL did not induce IL-17 production in vivo (data not shown). Therefore, the soluble FasL present in the FFL-sup was not responsible for the induction of IL-17. These results indicate that the FasL-induced IL-17 production is mediated by some secondary factor(s).

### *IL-1$\beta$ is partly responsible but not sufficient for the IL-17 production-inducing activity of FFL-sup*

The FasL-induced IL-17 production in vivo is severely but not completely diminished in IL-1-deficient mice, suggesting that IL-1 is involved in the FasL-induced IL-17 production (14). Therefore, we investigated whether IL-1$\beta$ is solely responsible for the IL-17 production induced by FFL-sup. The concentration of IL-1$\beta$ in FFL-sup ranged from 500 to 1000 pg/ml. Consistent with the result of the in vivo experiments described above, excess anti-IL-1$\beta$ Ab inhibited the in vitro IL-17 production induced by FFL-sup (Fig. 2*A*). However, rIL-1$\beta$ used at up to 10 ng/ml induced only a small amount of IL-17 (Fig. 2*B*). These results indicate that there was a factor that induced IL-17 production in synergy with IL-1$\beta$ in the FFL-sup.

### *IL-23 but not IL-15 is involved in the IL-17 production-inducing activity of FFL-sup*

It has been reported that IL-15 and IL-23 have the potential to induce IL-17 production (18, 25, 26). Thus, we tested whether these cytokines were involved in the IL-17-inducing activity of FFL-sup. A fusion protein consisting of the extracellular region of the IL-15 receptor and the Fc portion of IgG (IL15R-Fc) inhibited the ability of rIL-15 to induce IL-17 production in splenocytes (Fig. 3*A*). However, the same IL15-Fc failed to inhibit the activity of FFL-sup to induce IL-17 production (Fig. 3*B*). In contrast, the anti-IL-12 polyclonal Ab, which could neutralize both IL-12 and IL-23 (which share the common p40

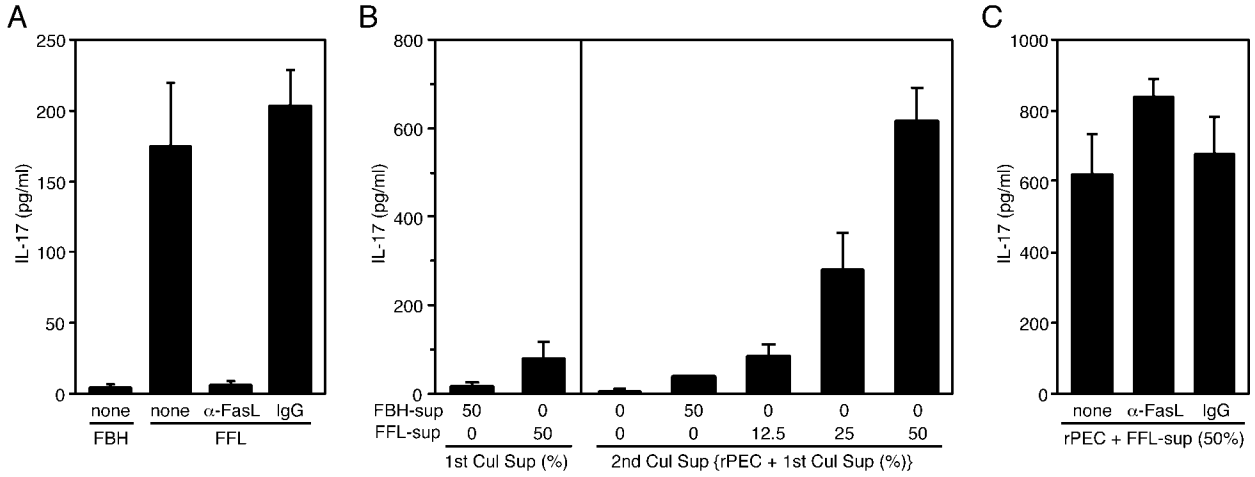


**FIGURE 1.** FasL induces the production of IL-17-inducing activity in resident PEC. *A*, Resident PECs ($5 \times 10^5$ cells) were cultured with $1 \times 10^5$ FBH or FFL cells in the presence or absence of anti-FasL mAb or normal hamster IgG, as indicated, for 20 h. The concentration of IL-17 in the culture supernatant is shown. *B*, Resident PECs were cultured with FBH or FFL cells as described in *A* (first culture). Freshly isolated resident PECs ($5 \times 10^5$ cells) were cultured for 20 h (second culture) in the presence of the indicated concentrations of the first culture supernatant (FBH- or FFL-sup). The concentration of IL-17 in the 2-fold diluted (50%) first culture supernatant (*left*) or in the second culture supernatant (*right*) is shown. *C*, Freshly isolated resident PECs ($5 \times 10^5$ cells) were cultured in 50% FFL-sup in the presence or absence of anti-FasL mAb or normal hamster IgG for 20 h. The concentration of IL-17 in the second culture supernatant is shown.

subunit), inhibited the FFL-sup-induced IL-17 production from resident PEC (Fig. 3*C*). In addition, rIL-23 but not IL-12 induced IL-17 production in resident PEC (Fig. 3*D*). These results strongly suggested that IL-23 played an important role in the IL-17 production induced by FFL-sup. Subsequently, we sought to confirm the presence of IL-23 in the FFL-sup by ELISA. As expected, a small but significant amount of p40 was detected in the FFL-sup but not in the FBH-sup (Fig. 3*E*). In contrast, we failed to detect IL-23 in the FFL-sup using sandwich ELISA in which anti-p19 mAb was used as the capture Ab and anti-p40 mAb was used as detection Ab (data not shown). However, the latter results could be due to low sensitivity of the IL-23-specific ELISA system. In fact, RT-PCR analysis indicated that the expression level of p19 mRNA in resident PECs was strongly enhanced in response to the stimulation by FFL but not FBH cells, whereas mRNAs for p40 and the IL-12 specific subunit p35 were moderately increased by the FFL stimulation (Fig. 3*F*, *left panels*). The induction of p19 mRNA was observed as early as after 8 h of FFL stimulation (Fig. 3*F*, *right panels*). These results suggest that IL-23 was present in the FFL-sup.

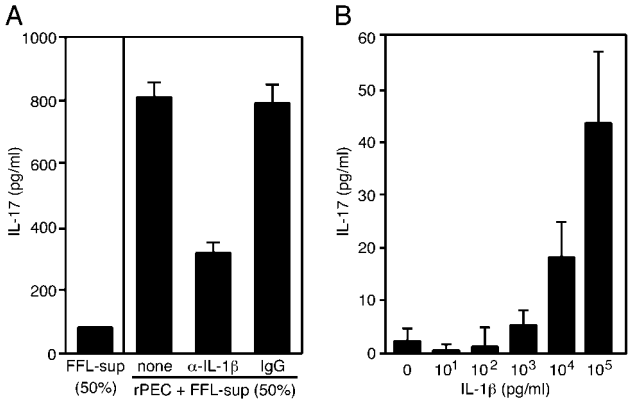


**FIGURE 2.** IL-1$\beta$ is involved in but not sufficient for potent IL-17 production. Resident PECs ($5 \times 10^5$ cells) were cultured in 50% FFL-sup with or without anti-IL-1$\beta$ polyclonal Abs or normal goat IgG for 20 h (*A*). Otherwise, resident PEC were cultured with the indicated concentrations of IL-1$\beta$ for 20 h (*B*). The concentration of IL-17 in the culture supernatant is shown.

### *IL-1$\beta$ and IL-23 synergistically induce IL-17 production in resident PECs*

Although the above results suggest that IL-23 was present in FFL-sup, the concentration of p40 in the FFL-sup was much lower than the concentration of rIL-23 required for potent IL-17 production, when rIL-23 acts alone. Furthermore, not only anti-IL-12 polyclonal Abs, but also the anti-IL-1$\beta$ mAb, inhibited the IL-17-inducing activity in FFL-sup (Figs. 2*A* and 3*E*). These results prompted us to investigate whether IL-1$\beta$ and IL-23 act in synergy to induce IL-17 production. As expected, IL-1$\beta$ and IL-23 cooperatively induced IL-17 production in resident PECs in vitro (Fig. 4, *A* and *B*). Addition of IL-12 did not enhance the IL-17 production induced by IL-1$\beta$ and/or IL-23 (Fig. 4*B*). Rather, IL-12 significantly inhibited the IL-23- or IL-1$\beta$ + IL-23-induced IL-17 production. This inhibition was probably because IL-12 competed with IL-23 to occupy IL-12R$\beta$1 that is the shared component of IL-12 and IL-23 receptors.

### *FasL induces IL-23, which is essential for the FasL-induced IL-17 production in vivo*

To investigate whether FasL induces the IL-23 produced in vivo, p40 in PLF and p19 and p40 mRNA in PECs prepared from mice 24 h after the injection of FBH or FFL cells were examined by ELISA and RT-PCR, respectively. The injection of FFL cells enhanced both p40 and p19 expression at protein and/or mRNA levels compared with the injection of FBH cells, whereas the expression levels of p35 mRNA were comparable between FFL- and FBH-PEC (Fig. 5, *A* and *B*). A significant amount of IL-23 was detected in FFL- but not FBH-PLF using an IL-23-specific ELISA, although the amount of IL-23 determined by the IL-23-specific ELISA was much lower than what we expected from p40 ELISA data (Fig. 5*C*). Because the FasL-induced IL-17 production was

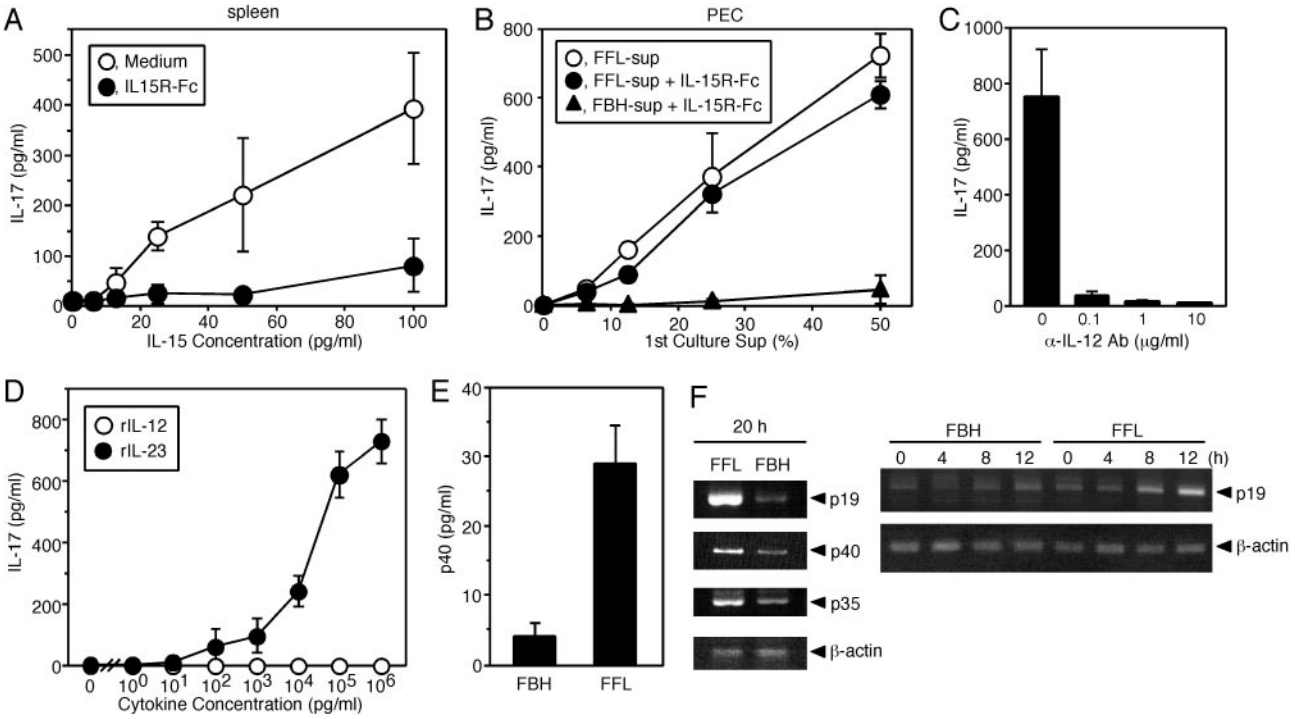


**FIGURE 3.** IL-23 but not IL-15 is involved in IL-17 production. *A*, Splenocytes ($1 \times 10^6$ cells) were cultured with the indicated concentrations of IL-15 in the presence or absence of IL-15R-Fc for 48 h. *B*, Resident PECs ($5 \times 10^5$ cells) were cultured with the indicated concentrations of FBH- or FFL-sup in the presence or absence of IL-15R-Fc for 20 h. *C*, Resident PECs ($5 \times 10^5$ cells) were cultured in 50% FBH- or FFL-sup in the presence of the indicated concentrations of anti-IL-12 polyclonal Abs for 20 h. *D*, Resident PECs ($5 \times 10^5$ cells) were cultured with the indicated concentrations of IL-12 or IL-23 for 20 h. *A–D*, The concentration of IL-17 in the culture supernatant is shown. *E* and *F*, Resident PECs ($5 \times 10^5$ cells) were cultured with $1 \times 10^5$ cells FFL or FBH cells for 20 h (*E*) or indicated period (*F*). The concentration of p40 in the culture supernatant (*E*) and the expression of p19, p40, p35, and β-actin mRNA in cells (*F*) after the culture is shown.

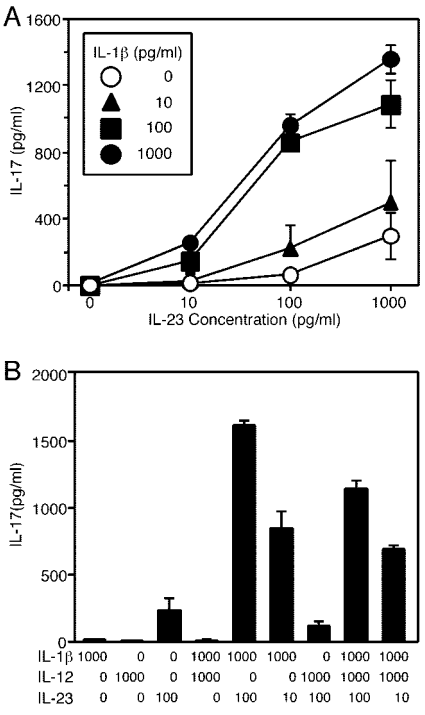


**FIGURE 4.** IL-1β and IL-23 synergistically induce IL-17 production in resident PECs. *A* and *B*, Resident PECs ($5 \times 10^5$ cells) were cultured with the indicated concentrations of IL-1β, IL-12, and/or IL-23 for 20 h. The concentration of IL-17 in the culture supernatant is shown.

severely impaired in IL-1-deficient mice, we tested whether FFL induced IL-23 production in IL-1-deficient mice. Comparable levels of p40 protein and p19 mRNA were produced upon FFL injection in wild-type and IL-1-deficient mice (Fig. 5, *A* and *B*). We then investigated whether IL-23 is involved in the FasL-induced IL-17 production in vivo using p40-deficient mice. As expected, no p40 was detected in the PLF from p40-deficient mice after FBH or FFL injection (Fig. 5*D*). Importantly, the FasL-induced IL-17 production was also severely impaired in the p40-deficient mice (Fig. 5*E*). Taken together, these results indicate that IL-23 was produced upon FasL stimulation independent of IL-1β, and IL-23 induced IL-17 production in synergy with IL-1β in vivo, consistent with our in vitro results.

### *BM-DCs produce IL-23 in response to FasL stimulation*

IL-23 is produced from DCs activated via CD40 (16). In addition, FasL is reported to induce the activation of rather than apoptosis in DCs (27). Therefore, we investigated whether FasL induces IL-23 production in DCs. DCs were generated by culturing BM cells in medium containing GM-CSF. On day 4 of culture, the majority of cells remained $CD11c^-I\text{-}A^-$, although a portion had become $CD11c^+$, and some even expressed I-A (Fig. 6*A*, *upper panel*). In contrast, on day 8, the proportion of $CD11c^+$ cells reached ~90% and most of the $CD11c^+$ cells expressed I-A (Fig. 6*A*, *lower panel*). Importantly, on day 8 BM-DCs produced p40, and stimulation by FFL cells enhanced its production (Fig. 6*B*). Furthermore, stimulation by FFL cells induced the expression of p19 mRNA as well as p35 and p40 mRNAs in the day 8 cells (Fig. 6*C*). An IL-23-specific ELISA

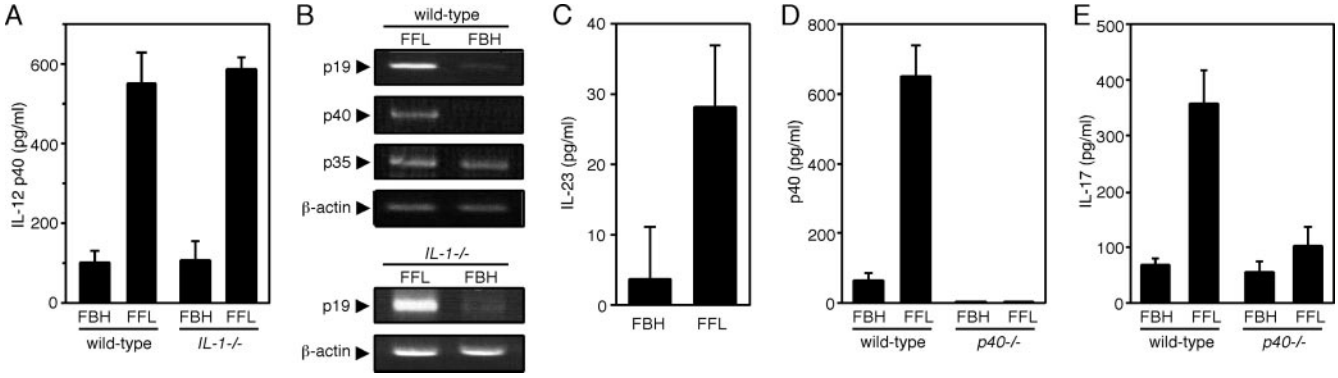


**FIGURE 5.** FasL induces IL-1$\beta$-independent IL-23 production in vivo, and the IL-23 is essential for the FasL-induced IL-17 production in vivo. *A–C*, FBH or FFL cells ($2 \times 10^6$ cells) were injected i.p. into wild-type or *IL-1*$^{-/-}$ mice, and PEC and PLF were obtained 20 h after the injection. The concentration of p40 in PLF (*A*), the expression of p19, p40, p35, and $\beta$-actin mRNA in the PECs (*B*), and the concentration of IL-23 in PLF (*C*) are shown. *D* and *E*, FBH or FFL cells ($2 \times 10^6$ cells) were injected i.p. into wild-type or *p40*$^{-/-}$ mice, and PLF was obtained 20 h after the injection. The concentration of p40 (*D*) or IL-17 (*E*) in PLF is shown.

also demonstrated that FasL stimulation induced IL-23 production, although the amount of IL-23 determined by the IL-23-specific ELISA was again much lower than what we expected from the p40 ELISA data (Fig. 6*D*). These results indicate that FasL induces IL-23 production in BM-DC. Because day 4 cells also produced p40, albeit at a lower level than the day 8 cells (Fig. 6*B*), and FFL cells stimulated p40 production, immature DC or DC precursor cells may be able to produce IL-23 and augment the IL-23 production in response to FasL.

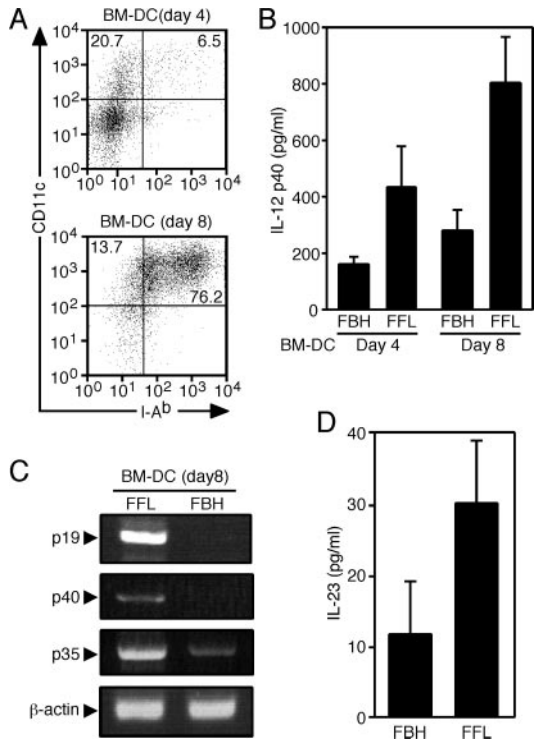


**FIGURE 6.** FasL induces IL-23 production in BM-derived DCs. *A*, BM cells were cultured with GM-CSF for 4 or 8 days. Expression of CD11c and I-A was examined by flow cytometry. *B* and *C*, The day 4 and day 8 BM-derived DCs described in *A* ($5 \times 10^5$ cells) were cultured with or without $1 \times 10^5$ FBH or FFL cells for 24 h. The concentration of p40 in the culture supernatant is shown in *B*. The expression of p19, p35, p40, and $\beta$-actin mRNA in cells (*C*) and the concentration of IL-23 in the culture supernatant (*D*) from day 8 BM-derived DCs cocultured with FBH or FFL are shown.

### *FFL-PECs produce IL-23 in response to FasL stimulation*

Resident PECs produced only a small amount of p40 upon FasL stimulation (Fig. 3*E*). In addition, 4-h thioglycolate-induced PECs, which were mainly neutrophils, produced no p40, and 4-day thioglycolate-induced PECs, which were mainly macrophages, produced only a small amount of p40 in response to FasL stimulation (Fig. 7*A*). In contrast, PECs isolated from mice that received an injection of mitomycin C-treated FFL cell (FFL-PECs) produced a large amount of p40 upon restimulation by FFL in vitro. FFL-PECs cultured with FBH cells produced p40 at ~50% of the level of the FFL-restimulated FFL-PECs. This was probably due to carryover of the in vivo-injected FFL cells in the culture. PEC isolated from mice that received a mitomycin C-treated FBH cell (FBH-PECs) injection also produced p40 at ~50% of the level of that produced by FFL-restimulated FFL-PEC, when the FBH-PEC cells were cultured with FFL cells. However, the number of FBH-PECs per mouse was ~40% of the number of FFL-PECs per mouse (FFL-PECs, $14.7 \pm 4.9 \times 10^6$ cells, $n = 10$; FBH-PECs, $6.3 \pm 2.1 \times 10^6$ cells; $n = 10$). Therefore, the total capacity of FFL-PECs to produce p40 was five times higher than the capacity of FBH-PECs. This ratio agrees with the ratio of p40 amounts in FFL- and FBH-PLF (Fig. 5*A*).

### *FFL-PEC-derived DCs produce IL-23 in response to FasL stimulation*

$CD11c^+$ cells were not a major population (~10%) in FFL-PEC (data not shown). To investigate whether DC precursor cells are present in FFL-PECs, FFL-PECs were cultured with GM-CSF for 3 days. After this relatively short period of culture, the majority of cells became $CD11c^+$, and a large portion of them expressed I-A (Fig. 7*B*), suggesting that FFL-PECs contained DC precursor cells. Furthermore, restimulation of FFL-PEC-derived DCs with FFL cells enhanced p40 production and p19 as well as p40 and p35 mRNA expression in vitro (Fig. 7, *C* and *D*). Thus, the DC and DC precursor cells among the FFL-PEC are candidates for IL-23 production.

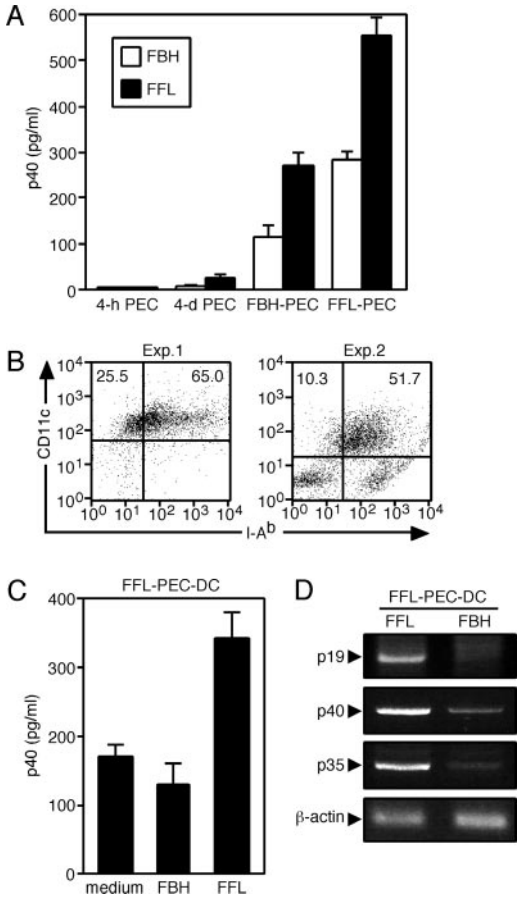


**FIGURE 7.** FasL induces p40 production in PEC-derived DCs. *A*, PECs were prepared from mice treated with thioglycolate medium for 4 h (4-h PECs) or 4 days (4-day PECs) or from mice that received an i.p. injection of mitomycin C-treated FBH or FFL cells 20 h before the PEC harvest (FBH- or FFL-PECs). The PECs ($5 \times 10^5$ cells) were cultured with $1 \times 10^5$ FBH (□) or FFL cells (■) for 24 h. The concentration of p40 in the culture supernatant is shown. *B*, The FFL-PECs described in *A* were cultured with GM-CSF for 3 days. The proportion of $CD11c^+I\text{-}A^-$ and $CD11c^+I\text{-}A^+$ cells was determined by flow cytometry. The data from two independent experiments are shown. *C* and *D*, The FFL-PEC-derived DCs described in *B* ($5 \times 10^5$ cells) were cultured with or without $1 \times 10^5$ FBH or FFL cells for 24 h. The concentration of p40 in the culture supernatant (*C*) and the expression of p19, p40, p35, and β-actin mRNA in cultured cells (*D*) are shown.

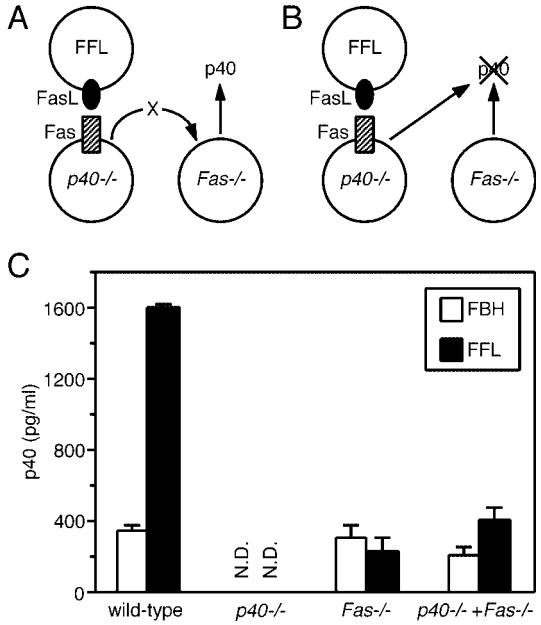


**FIGURE 8.** FasL induces p40 production in a cell-autonomous manner. *A* and *B*, Experimental design. If FasL induces some factor(s) (*X* in model *A*) that is capable of inducing IL-23 production in a paracrine manner, a mixture of $p40^{-/-}$ and $Fas^{-/-}$ DCs would produce IL-23 upon FasL stimulation, as shown in *A*. If FasL induces IL-23 production in a cell-autonomous manner, neither $p40^{-/-}$ nor $Fas^{-/-}$ DCs would produce p40 upon FasL stimulation, as shown in *B*. *C*, Wild-type DCs, $p40^{-/-}$ DCs, $Fas^{-/-}$ DCs, or a mixture of $p40^{-/-}$ DCs and $Fas^{-/-}$ DCs ($5 \times 10^5$ cells) were cultured with $1 \times 10^5$ FFL or FBH cells for 24 h. The concentration of p40 in the culture supernatants is shown.

### *FasL induces cell-autonomous p40 production in DC*

A remaining important question was whether FasL directly induces IL-23 production in DCs. It was possible that some factors that were produced upon FasL stimulation secondarily induced p40 production. To discriminate between these possibilities, we mixed BM-DCs prepared from $p40^{-/-}$ mice and those derived from $Fas^{-/-}$ mice and cocultured them with FFL cells. If FasL induced a factor that in turn induced p40 production in a paracrine manner, p40 production would be induced by FFL under these conditions (Fig. 8*A*), whereas if FasL only directly induced p40 production, then p40 production would not be induced under these conditions (Fig. 8*B*). The controls were $p40^{-/-}$ DCs, which produced no p40, and $Fas^{-/-}$ DCs stimulated with FFL, which produced a basal level of p40 that was also observed when the $Fas^{-/-}$ DCs were cultured with FBH (Fig. 8*C*). The latter result confirmed that the FasL-Fas interaction induced p40 production in DCs. Finally, the mixture of $p40^{-/-}$ DCs and $Fas^{-/-}$ DCs produced only a small amount of p40 in response to FFL stimulation. These results indicate that the FasL-induced p40 production is mainly a cell-autonomous response.

## Discussion

We previously demonstrated that peritoneal $CD4^-CD8^-$ TCRαβ or γδ T cells produce IL-17 in response to FasL stimulation in vivo, and this response was severely diminished in IL-1-deficient mice (14). Consistent with this, FasL-induced IL-17 production in vitro was inhibited by anti-IL-1β Ab. However, IL-1β alone induced only weak IL-17 production. This apparent discrepancy can be explained by our new finding that IL-1β dramatically enhanced IL-23-induced IL-17 production. Furthermore, FFL-induced IL-17 production was severely impaired in p40-deficient mice. Consistent with a previous report (19), IL-12, unlike IL-23, did not induce IL-17 production in resident PECs. These results strongly suggest that the FasL-induced IL-17 production is mediated by the synergistic action of IL-1β and IL-23, although this idea needs to be confirmed using mice deficient in p19, a specific component of IL-23.

It has been reported that DC and macrophages are the major producers of IL-23. In this study, we showed that resident PECs produced only a small (but sufficient to induce potent IL-17 production in the presence of IL-1β) amount of IL-23 in response to FasL stimulation in vitro. Thioglycolate-induced peritoneal exudate neutrophils and macrophages were also found to be poor producers of IL-23 in response to FasL. In contrast, BM-DC generated after 8 days of GM-CSF stimulation produced a large amount of p40 and expressed p19 mRNA upon

FasL stimulation. Interestingly, BM cells after 4 days of GM-CSF stimulation, which mainly remained $CD11c^{-}I\text{-}A^{-}$, produced p40 at ~50% the level of day 8 BM-DC. These results are consistent with a recent report by Rescigno et al. (27) showing that stimulation by FasL or anti-Fas Ab did not induce apoptosis in immature and mature DCs, but rather elicited the maturation and/or activation of these cells.

We also found that FFL-PECs produced IL-23 in vitro, although the majority of FFL-PECs are neutrophils (23) and $CD11c^{+}$ cells were not a major population (~10%) in FFL-PECs (data not shown). However, when FFL-PECs were cultured with GM-CSF for only 3 days, the neutrophils disappeared, probably by apoptosis, and the majority of the remaining cells became $CD11c^{+}I\text{-}A^{+}$ cells. This quick development of DCs from FFL-PECs suggests that the FFL-PECs contained a significant number of DC precursor cells. Based on these considerations, we would suggest that these DC precursor cells were the IL-23 producers in the FFL-PECs. Because resident PEC were poor producers of IL-23, such DC precursor cells might be derived from blood monocytes that migrated into the peritoneal cavity upon FFL transplantation. Consistent with this idea, we have found that except for $Gr\text{-}1^{high}$ neutrophils, $Mac\text{-}1^{high}Gr\text{-}1^{low}$ macrophage/monocyte-like cells are a major population in FFL-PECs and that the majority of $CD11c^{+}$ cells in FFL-PECs were the $Gr\text{-}1^{low}$ cells (data not shown). $B220^{+}CD11c^{+}$ plasmacytoid DCs and $CD4^{+}CD11c^{+}$ lymphoid DCs were not significantly present in FFL-PECs (data not shown), although we cannot rule out a possibility that such DC subsets may also have a capacity to produce IL-23 upon FasL stimulation. Alternatively, it is possible that IL-23 producers, which might or might not belong to the DC lineage, existed in the peritoneal cavity, but were tightly attached to the peritoneal wall and thus were not efficiently included in the isolated resident PECs. Such IL-23 producers might be mobilized upon FFL transplantation and, therefore, included in the FFL-PEC population.

We previously discovered that FasL induces apoptosis and IL-1$\beta$ secretion in neutrophils; both of these responses are mediated by caspases (7). IL-18 is secreted by macrophages by a similar mechanism (13). However, others (14, 15) and we have found that FasL induces the production of other inflammatory cytokines and chemokines that do not require caspases for their posttranscriptional maturation and include IL-6, IL-17, KC, and MIP-2 in mice. IL-23 is a new member of the FasL-inducible cytokines. Some of these cytokines may be produced by indirect mechanisms. In fact, we showed here that FasL-induced IL-17 production is mediated by IL-1$\beta$ and IL-23. FasL-induced KC production is reduced in IL-1-deficient mice, and the administration of anti-IL-17 mAb into IL-1-deficient mice further diminishes the FasL-induced KC production (14). Thus, FasL-induced KC production is mediated by IL-1$\beta$ and IL-17. In contrast, as demonstrated in this study, p40 production is largely a cell-autonomous response to FasL stimulation. FasL induced p19 mRNA expression, and therefore FasL induced IL-23 production, at least in part, at the mRNA level. We recently demonstrated that FasL induces IL-8 production in the HEK293 human cell line through cell-autonomous NF-$\kappa$B activation, in which caspase-8 plays an essential role (28). FasL also induces AP-1 transcriptional activity in HEK293 cells (28). Thus, FasL induces cell autonomous cytokine production in two ways: in one, it induces caspase-dependent maturation of cytokine precursors such as pro-IL-1$\beta$ and pro-IL-18; in the other, it induces the expression of cytokine mRNAs—this induction also, at least in part, depends on a caspase.

IL-23 and IL-23R are structurally related to IL-12 and IL-12R, respectively (16, 17). Initially, it was suggested that the biological functions of IL-12 and IL-23 are similar. However, recent studies (20, 21) involving IL-23 transgenic mice or IL-23-deficient mice showed that IL-23 plays important roles in the effector phase of inflammation rather than in the Th1 polarization of the immune response. Thus, the axis of FasL-IL-23 is a novel cytokine pathway of FasL-induced inflammation that is in addition to the FasL-IL-1$\beta$ pathway. The next question is whether mice deficient for both IL-1$\beta$ and IL-23 are completely free from FasL-induced inflammation. If so, FasL may easily confer immune privilege in such mice.

## Disclosures

The authors have no financial conflict of interest.

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