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Induction of Rapid T Cell Death and Phagocytic Activity by Fas-Deficient lpr Macrophages

Ritsuko Oura,*† Rieko Arakaki,* Akiko Yamada,* Yasusei Kudo,* Eiji Tanaka,† Yoshio Hayashi,* and Naozumi Ishimaru*

Peripheral T cells are maintained by the apoptosis of activated T cells through the Fas–Fas ligand system. Although it is well known that normal T cells fail to survive in the Fas-deficient immune condition, the molecular mechanism for the phenomenon has yet to be elucidated. In this study, we demonstrate that rapid cell death and clearance of normal T cells were induced by Fas-deficient lpr macrophages. Transfer of normal T cells into lpr mice revealed that Fas expression on donor T cells was promptly enhanced through the IFN-γ/IFN-γR pathway. In addition, Fas ligand expression and phagocytic activity of lpr macrophages were promoted through increased NF-κB activation. Controlling Fas expression on macrophages plays an essential role in maintaining T cell homeostasis in the peripheral immune system. Our data suggest a critical implication to the therapeutic strategies such as transplantation and immunotherapy for immune disorder or autoimmunity related to abnormal Fas expression.

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Abbreviations used in this article: AICD, activation-induced cell death; B6, C57BL/6; B6/lpr, C57BL/6/lpr; B6/gld, C57BL/6/gld; B6/lpr/gld, C57BL/6/lpr/gld; FasL, Fas ligand; IFN-γR<sup>−/−</sup>, IFN-γ receptor gene knockout; LN, lymph node; PEC, peritoneal exudate cell; TG, transgenic.

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Gy before T cell transfer. For the analysis of donor T cells, spleen cells, lymph node (LN) cells, PBMCs, or peritoneal exudate cells (PECs) were analyzed by flow cytometry. To inhibit in vivo deletion of T cells, anti-Fasl mAb (clone MFL3; BioLegend, San Diego, CA) was i.p. injected into recipient mice together with transfer of T cells.

Flow cytometry
FITC, PE, allopurinol–peridin chlorophyll protein, PE-Cy5.5, PE-Cy7, or allopurinol–Cy7-conjugated Abs including anti-CD4, CD8, CD11b, Fas, and Fasl Abs, were used. A FACSscan flow cytometer (BD Biosciences, Franklin Lakes, NJ) was used, and data were analyzed using the FlowJo FACS Analysis software (Tree Star, Ashland, OR).

In vivo imaging
The mice were s.c. injected with isoflurane (Abbott Laboratories, Abbott Park, IL) and transferred using a precise Micropump (Caliper Life Sciences, Hopkinton, MA) for 30 min. A total of 5 × 10⁶ T cells were i.v. transferred into recipient mice, and donor T cells were monitored at 30 min, 2 h, and 6 h using in vivo imaging analyzer (Caliper Life Sciences).

ELISA
The concentration of IFN-γ in sera was measured by ELISA. Ninety-six-well flat-bottom plates were precoated with capture Abs, and diluted samples or standard recombinant cytokines were added to each well. After the plates were washed, biotinylated Abs were added, and the wells were incubated with HRP-labeled, affinity-purified anti-rat IgG. A solution of 3,3’-5,5’-tetramethylbenzidine was used as a substrate. The absorbance of the samples was measured using a microplate reader (Model 680; Bio-Rad Laboratories, Richmond, CA).

Quantitative RT-PCR
Total RNA was extracted from spleen cells or PECs using Isogen (Wako Pure Chemical Industries, Osaka, Japan); it was then reverse transcribed. The transcript levels of FasL, TNF-α, IL-6, IL-1β, and β-actin were performed using a PTC-200 DNA Engine Cycler (Bio-Rad Laboratories) with SYBR Premix Ex Taq (Takara Bio, Shiga, Japan). The primer sequences used were as follows: FasL, forward, 5′-GGGCGGCTCCTAGGACCA-3′, and reverse, 5′-CTCTGCAAGAGACTGACGTC-3′; TNF-α, forward, 5′-ATAGACACAGAAACGATAGC-3′, and reverse, 5′-AGATGACTGATGTTGAGG-3′; IL-6, forward, 5′-CTTGGCAAGAGACCTTCCAT-3′, and reverse, 5′-ATGGCAAAATTTCTGATTATA-3′; IL-1β, forward, 5′-TGATGAGAATGACTGCTTCT-3′, and reverse, 5′-CTTCTCAAAAGTGAGAAGAAA-3′; β-actin, forward, 5′-GGTGGCCGCTCCTAGGACCA-3′, and reverse, 5′-GGTGGCCGCTCCTAGGACCA-3′.

Preparation of peripheral macrophages
Mice were i.p. injected with 1 ml 3% thioglycollate broth (Sigma-Aldrich), and after 3 or 4 d, elicited macrophages were collected by peritoneal lavage with 5 ml ice-cold PBS.

Phagocytosis assay
Phagocytosis was assessed using FlowNebrate Yellow Green Caboxylate Microspheres (Polysciences, Warrington, PA). Briefly, the CD11b⁺ cells purified from PECs were incubated with opsonized beads for 30 min at 37°C and washed with PBS. The phagocytic activity of CD11b⁺ cells was evaluated by flow cytometric analysis.

Apoptosis detection assay
Apoptosis was detected using the Annexin V-FITC apoptosis detection kit (BioVision, Mountain View, CA). Briefly, the cells were washed with PBS and stained with FITC-conjugated annexin V and propidium iodide for 15 min at room temperature in the dark. Binding buffer was added, and apoptosis cells were detected by flow cytometric analysis. To inhibit in vitro T cell apoptosis cocultured with PECs, PECs were treated with a Fas-Fc fusion protein (R&D Systems, Minneapolis, MN).

Confocal microscopic analysis
PECs including GFP⁺ T cells were stained with PE-conjugated anti-CD11b mAb (eBioscience) on a glass slide. Coverslips were applied with Fluoromount-G (Molecular Probe). Cells were visualized using a Confocal Laser Microscan (LSM 5 Pascal; Carl Zeiss, Oberkochen, Germany).

Western blot analysis
Cell extracts from the nucleus and cytoplasm of CD11b⁺ PECs were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Rockford, IL). A total of 10 µg of each sample per well was used for SDS-PAGE. After blocking with 5% nonfat milk, the membrane was incubated with primary Abs against phospho-IκBα and p50 (NF-κB1), RelA (p65), and histones (Santa Cruz Biotechnology, Santa Cruz, CA). Ag–Ab complexes were detected using HRP-conjugated secondary Abs. Protein binding was visualized using the Phototope-HRP Western blot Detection System (Cell Signaling Technology, Danvers, MA).

NF-κB transcription activity assay
The transcription activity of NF-κB in the nuclear extracts from PECs was analyzed with a NF-κB transcription factor colorimetric assay kit (Millipore, Billerica, MA). Briefly, nuclear extracts were incubated with a biotinylated double-stranded oligonucleotide probe containing the consensus sequence for NF-κB on a streptavidin-coated plate. Captured complexes, including active NF-κB protein, were incubated with the primary Abs for p50 and RelA, HRP-conjugated secondary Ab, and tetramethylbenzidine substrate. The absorbance of the samples was measured using a microplate reader at 450 nm.

Statistical analysis
Statistical significance was determined with an unpaired Student t test.

Results
Normal T cell dynamics in Fas-deficient mice
To understand the dynamics of normal T cells in Fas-deficient mice, the T cells from GFP-TG mice were i.v. transferred into B6 and B6/lpr mice. On 7 d after the transfer, GFP⁺ cells in the spleen and LNs of the recipient mice were analyzed. Although GFP⁺ T cells were found in the spleen and LNs of B6 mice, these cells were barely detectable in B6/lpr mice (Fig. 1A). To evaluate the in vivo expansion of normal T cells in Fas-deficient mice using a homoeostatic proliferation system, CFSE-labeled normal T cells from B6 mice were i.v. transferred into irradiated B6 and B6/lpr mice. On 7 d after the transfer, expanded T cells were found in B6 mice (Fig. 1B). However, the transferred CFSE⁺ T cells in the spleen and LNs of B6/lpr mice were almost undetectable (Fig. 1B). In addition, to examine the in vivo Ag-specific T cell response in B6/lpr mice, CD4⁺ T cells were purified from the spleen of OVA-specific TCR-TG (OT-II) mice and were transferred into B6 and B6/lpr mice. OVA peptide (100 µg/mouse) was injected into the recipient mice on the following day. On 7 d after the transfer, OT-II-specific Vβ5.2⁺CD4⁺ T cells of the spleen and LNs were analyzed. Although OT-II T cells were found in the spleen and LNs of B6 recipients, these cells were almost undetectable in the spleen and LNs of B6/lpr mice (Fig. 1C). These findings indicate that normal T cells fail to migrate to lymphoid tissues or survive under the Fas-deficient environment. In addition, we examined whether transferred T cells migrate to any specific organ other than lymphoid organs. On 7 d after the transfer of GFP⁺ T cells, T cell diminishment was observed in the spleen, LNs, and liver of B6/lpr recipient mice (Fig. 1D). The accumulation of the donor T cells was not observed in any specific organs such as the lung, pancreas, and kidney. Furthermore, the donor T cells did not accumulate in the thymus, bone marrow, and PBMCs of B6/lpr recipient mice (Fig. 1D). These findings demonstrate that transferred normal T cells fail to survive in the lymphoid organs such as the spleen and LNs of B6/lpr recipients.

Migratory response of normal T cells in Fas-deficient recipients
To examine the migration of normal T cells to lymphoid tissues, in vivo imaging analysis of the dynamics of normal T cells in B6 and B6/lpr mice was performed. At 30 min after the transfer of
were detectable in both the lung and liver after i.v. injection of T cells. Normal T cells were detectable in the spleen and LNs of recipient mice at day 7 after the transfer were detected by flow cytometry. Normal T cells (Thy1.1+) were analyzed 30 min after the transfer, the rapid diminishment of T cells in B6/lpr recipients had already been observed (Fig. 2B). This suggests that rapid death and clearance of normal T cells may have occurred in the B6/lpr recipients immediately after the transfer. Moreover, when the Fas expression on the transferred normal T cells in B6 or B6/lpr recipients was analyzed 30 min after the transfer, significantly increased Fas expression was observed on the T cells in B6/lpr recipients compared with those in B6 recipients (Fig. 2C). Furthermore, when CFSE-labeled T cells from B6/lpr mice were i.v. injected into B6 and B6/lpr mice, the T cell diminishment was not detectable in both recipient mice (Fig. 2D). These results suggest that Fas expression on normal T cells plays a crucial role in the induction of rapid T cells diminishment in Fas-deficient recipients. These results imply that Fas-mediated cell death of normal T cells is enhanced in a Fas-deficient immune environment.

**FasL expression on immune cells in B6/lpr mice**

Next, we analyzed FasL expression on peripheral immune cells in B6/lpr mice. When mRNA expression of FasL in the spleen of B6 and B6/lpr mice was analyzed by quantitative reverse transcription-PCR (RT-PCR), higher levels of FasL mRNA of all subsets including CD4+ T cells, CD8+ T cells, CD11c+ dendritic cells, and CD11b+ macrophages were observed in B6/lpr mice compared with those in B6 mice (Fig. 3A). In addition, significantly increased FasL mRNA expression of subsets including CD4+ T cells, CD8+ T cells, and CD11b+ macrophages was observed in PBMCs from B6/lpr mice compared with those of B6 mice (Fig. 3B). Therefore, we speculated that FasL–Fas-mediated apoptosis of normal T cells may be induced by the interaction with the peripheral immune cells in B6/lpr mice. In particular, because FasL mRNA expression on the CD11b+ macrophages in the spleen and PBMCs of B6/lpr mice was much higher than that of B6 mice, macrophages may play a crucial role in T cell apoptosis and clearance in the immune system of B6/lpr mice. Thus, we focused on analyzing the macrophages in B6/lpr mice. When the surface expression of FasL on macrophages was analyzed by flow cytometry, the expression on the CD11b+ macrophages of the PBMCs of B6/lpr mice was increased compared with that of B6 mice (Fig. 3C). These results suggest that increased FasL expression on immune cells, including macrophages in a Fas-deficient immune condition, play an important role in the rapid diminishment of normal T cells.
role in the Fas-mediated rapid death of normal T cells in B6/lpr mice.

Functions of macrophages in B6/lpr mice

To understand the functions of the peripheral macrophages in B6/lpr mice, macrophages from thioglycolate-elicited PECs were used for analyzing their interaction with normal T cells. When the expression level of FasL on PECs was analyzed, significantly increased expression of FasL on PECs from B6/lpr mice was detected compared with B6 mice (Fig. 4A). Next, we examined whether in vitro T cell death was induced by coculture with PECs from B6/lpr mice. The proportion of apoptotic T cells cocultured with B6/lpr PECs was significantly enhanced compared with that of normal T cells cocultured with B6 PECs (Fig. 4B). To determine whether B6/lpr PECs engulf dead T cells rapidly, CFSE-labeled normal T cells were cocultured with CD11b+ PECs for 3 h, and CD11b+CFSE+ macrophages engulfing apoptotic T cells were analyzed. We detected a significant increase of CFSE+ CD11b+ macrophages in B6/lpr mice compared with B6 mice (Fig. 4C). In addition, we investigated the phagocytic activity of B6/lpr macrophages with FITC-labeled latex beads. The phagocytic activity of CD11b+ PECs in B6/lpr mice was significantly increased compared with that in B6 mice (Fig. 4D). To rule out that the apoptotic cells attached to macrophages, we fixed the macrophages after phagocytosis assay and then treated them with 0.01% Triton X-100. Because there was no change in the proportion of apoptotic cells attached to macrophages, we fixed the macrophages with FITC-labeled latex beads. The phagocytic activity of CD11b+ macrophages in vitro. B6 and B6/lpr mice were analyzed by quantitative RT-PCR. Data are shown as mean ± SD for five mice in each recipient group. *p < 0.05, **p < 0.005. (B) FasL mRNA expressions in PBMCs of B6 and B6/lpr mice were analyzed by quantitative RT-PCR. Data are shown as mean ± SD for five mice in each recipient group. *p < 0.05. (C) FasL expression on the CD11b+ macrophages in PBMCs was detected by flow cytometry. Results are representative of three independent experiments.

In vivo functions of Fas-deficient macrophages

To determine the in vivo functions of macrophages in B6/lpr mice, normal T cells from GFP-TG mice were i.p. injected into the recipient mice that had been injected with thioglycolate. At 6 h after T cell injection, PECs including injected T cells were analyzed. Consistent with the results obtained from the i.v. injection of normal T cells into B6/lpr mice, the T cells injected (i.p.) into B6/lpr mice were significantly decreased compared with those injected into B6 mice (Fig. 5A). The number of apoptotic cells showing Annexin V+ of injected T cells in B6/lpr mice was significantly higher compared with that in B6 mice (Fig. 5B). The depletion of T cells in B6/lpr recipients was inhibited by i.p. injection of anti-Fas mAb (Fig. 5C). Furthermore, when normal

FIGURE 4. Enhanced FasL expression and phagocytic activity of lpr macrophages in vitro. B6 and B6/lpr mice were i.p. injected with thioglycolate to obtain PECs. On day 4 after the injection, PECs were collected from peritoneal cavity. (A) FasL expression on CD11b+ macrophages in PECs of B6 and B6/lpr mice was analyzed by flow cytometry. Data are representative of four independent experiments. (B) CFSE-labeled T cells from Thy1.1 B6 mice were cocultured with the CD11b+ macrophages from PECs of B6 and B6/lpr mice. Annexin V+ apoptotic T cells (percentage) are shown as mean ± SD for five mice in each group. *p < 0.05. (C) CFSE-labeled T cells from B6 mice were cocultured with the CD11b+ macrophages from PECs of B6 and B6/lpr mice. Phagocytosis of CFSE+T cells by the CD11b+ cells in PECs was evaluated by flow cytometry. Results are shown as mean ± SD for five mice in each group. **p < 0.005. (D) Phagocytic activity of CD11b+ macrophages in PECs was evaluated by uptake of FITC-conjugated beads in vitro. Results are shown as mean ± SD for five mice in each group. **p < 0.005.
without Fas-Fc (Fig. 5D). In addition, the phagocytic activity of CD11b+ PECs in B6/lpr mice was significantly enhanced compared to that in B6/mice (Fig. 5E). Furthermore, microscopic analysis showed fewer normal T cells (GFP+) in B6/lpr mice compared with B6 mice: moreover, it revealed phagocytic fragments of GFP+ T cells within the macrophages in B6/lpr mice, although the fragments within the macrophages in B6 mice were undetectable (Fig. 5F). In contrast, when normal T cells from GFP-TG mice were i.p. injected into B6 and B6/gld mice, which are deficient in FasL expression, T cell diminishment was not observed (Fig. 5G). In contrast, we have performed the experiment using purified B cells. Because deletion of normal B cells in lpr recipients was not observed (Supplemental Fig. 3), T cell apoptosis may be closely associated with phagocytosis of lpr macrophages. Our findings reveal that Fas-deficient macrophages can induce rapid apoptosis through upregulated Fasl, and that Fas-deficient macrophages rapidly engulf apoptotic T cells.

Enhanced activation of Fas-deficient macrophages through NF-κB

NF-κB signaling plays a crucial role in the activation of macrophages (22, 23). Phosphorylation of IκBα, an endogenous inhibitory molecule of NF-κB activation by the interaction with NF-κB subunits, in CD11b+PECs from B6/lpr mice was much higher than that in CD11b+ PECs from B6 mice (Fig. 6A). Moreover, nuclear transport of NF-κB subunits such as p50 and p65 in CD11b+ PECs from B6/lpr mice was significantly enhanced compared with that in CD11b+ PECs from B6 mice (Fig. 6A). In addition, the transcriptional activity of NF-κB in the nuclear protein of the CD11b+ PECs from B6/lpr mice was significantly increased compared with that from B6 mice (Fig. 6A). In immune cells, including macrophages, there are several genes regulated by NF-κB such as TNF-α, IL-6, IL-1β, and FasL in immune cells including macrophage (24–27). The mRNA expression of TNF-α, IL-6, and IL-1β in CD11b+ PECs from B6/lpr mice was not increased compared with that from B6 mice (Fig. 6C). In contrast, the Fasl mRNA level in the CD11b+ PECs from B6/lpr mice was significantly higher than that from B6 mice (Fig. 6D). When Fasl mRNA of the CD11b+ PECs from NF-κB mice was knocked out mice bearing a fas gene mutant (NF-κB−/−/lpr) was analyzed, the expression level was similar to that of B6 mice (Fig. 6D). Furthermore, the diminishment of normal T cells in NF-κB−/−/lpr mice was not observed (Fig. 6E, 6F). These results suggest that FasL overexpression through NF-κB activation of macrophages is important for rapid T cell death in a Fas-deficient immune system.

Alteration of Fas expression on normal T cells in a Fas-deficient immune system

Fas expression is regulated by several factors or signaling pathways (28–31). One potent factor that induces Fas expression is IFN-γ (28). When the serum level of IFN-γ was analyzed by ELISA, we found that the concentration of the sera in B6/lpr mice was significantly higher compared with that in B6 mice (Fig. 7A). To determine the source of the high level of IFN-γ, subsets of peripheral immune cells including CD4+ T cells, CD8+ T cells, CD11b+ macrophages, CD11c+ dendritic cells, and B220+ B cells in PBMCs were purified, and IFN-γ mRNA was quantified by quantitative RT-PCR. The mRNA levels in CD4+, CD8+ T, and B220+ B cells from B6/lpr mice increased significantly compared with those in B6 mice (Fig. 7B). When the T cells from normal mice or IFNγR−/− mice were labeled with CFSE and were i.v. injected into B6/lpr mice, Fas expression on T cells in IFNγR−/− mice was not enhanced, although the expression on T cells from B6 mice was considerably increased in Fas-deficient recipients.

FIGURE 5. In vivo rapid death and phagocytosis of T cells by macrophages. (A) T cells (1 × 10⁶) from GFP-TG mice were i.p. injected into the recipient mice treated with thioglycolate. GFP+ T cells in PECs were detected by flow cytometry. Data are representative of four mice in each recipient group. (B) Annexin V+ apoptotic cells (percentage) of GFP+ T cells in PECs were detected by flow cytometry. Data are representative of four mice in each recipient group. (C) GFP T cells (5 × 10⁵) were cocultured with B6/lpr PECs (2 × 10⁷) for 24 h in vitro in the presence of Fas-Fc fusion protein (0, 0.1, and 5 μg/ml). GFP+ survival cells are shown as mean ± SD for triplicates. (D) Phagocytosis of GFP+ T cells by the CD11b+ macrophages in PECs was evaluated by flow cytometry. Data are representative of four mice in each recipient group. (E) GFP+ T cells (green) and CD11b+ macrophages (red) in PECs were detected by confocal microscopy. Original magnification ×630. Data are representative of four independent experiments. (F) T cells from GFP-TG mice were i.p. injected into B6 and B6/gld mice treated with thioglycolate. GFP+ T cells in PECs were detected by flow cytometry. Data are representative of four mice in each recipient group. GFP-T cells were i.p. transferred into B6/lpr mice together with Fas-Fc fusion protein (0.1 and 5 μg/ml), survival T cells were significantly increased in comparison with those of the recipients.
FIGURE 6. Control of FasL expression on lpr macrophages by NF-κB activation. (A) Phosphorylation of IkBα and nuclear translocation of the NF-κB subunits of CD11b+ macrophages from thioglycollate-induced PECs were analyzed by Western blotting. GAPDH and histones were used as housekeeping proteins. Data are representative of three independent experiments. (B) Transcriptional activity of NF-κB in B6 and B6/lpr macrophages was detected. Results are shown as mean ± SD for five mice in each group. *p < 0.005. (C) The mRNA expression of NF-κB-target genes was analyzed by quantitative RT-PCR. Data are shown as mean ± SD for five mice in each group. (D) FasL mRNA expression in the CD11b+ macrophages from thioglycollate-induced PECs in B6, B6/lpr, and NF-κBβ2/lpr mice was analyzed by quantitative RT-PCR. Data are shown as mean ± SD for five mice in each group. **p < 0.05. (E) IFN-γ mRNA expressions in the subsets of spleen cells from B6 and B6/lpr mice were analyzed by quantitative RT-PCR. Results are shown as mean ± SD for five mice in each group. *p < 0.05. (F) T cells from GFP-TG mice were i.p. injected into B6, B6/lpr, and NF-κBβ2/lpr mice pretreated with thioglycollate. GFP+ T cells in PECs were detected by flow cytometry. Data are representative of five mice in each group. (**p < 0.005. (G) GFP+ T cells (green) and CD11b+ macrophages (red) in PECs were detected by confocal microscopy. Original magnification ×630. Photos are representative of four independent experiments.

(Fig. 7C). Fas expression on T cells was enhanced by recombinant IFN-γ in a dose-dependent manner (Supplemental Fig. 4). In addition, when T cells from IFNγR−/− mice were i.v. injected into B6/lpr mice, T cell diminishment, which had been observed in the Fas-deficient recipients, was not detectable (Fig. 7D). Moreover, when T cells from IFNγR−/− mice were cocultured with lpr PECs, survival T cells of IFNγR−/− mice were significantly increased compared with those of wild-type mice (Fig. 7E). By the

FIGURE 7. Regulation of Fas expression on donor T cells by IFN-γ in lpr mice. (A) Concentration of IFN-γ in the sera of B6 and B6/lpr mice was measured by ELISA. Results are shown as mean ± SD for six mice in each group. **p < 0.005. (B) IFN-γ mRNA expression in the subsets of spleen cells from B6 and B6/lpr mice was analyzed by quantitative RT-PCR. Results are shown as mean ± SD for five mice in each group. *p < 0.005. (C) CFSE-labeled T cells from B6 and IFNγR−/− mice were i.v. injected into B6/lpr mice. Fas expression on the donor T cells in PBMCs of recipients was analyzed by flow cytometry. Data are representative of five mice in each group. Gray shadow shows isotype negative control. (D) CFSE-labeled T cells from B6 and IFNγR−/− mice were i.v. injected into B6 or B6/lpr mice and were detected by flow cytometry. Data are representative of four mice in each group. (E) CFSE-labeled T cells from B6 and IFNγR−/− mice were i.p. injected into thioglycollate-treated B6 or B6/lpr mice. CFSE+ T cells in PECs were analyzed by flow cytometry. Data are representative of four mice in each group. (F) T cells (2 × 10^5) from B6 mice were cocultured for 8 h with B6 or B6/lpr PECs (1 × 10^5) in the presence of anti–IFN-γ mAb (0, 2, 5, and 10 ng/ml). Apoptotic T cells were evaluated by flow cytometric analysis of expression of Annexin V. Results are shown as mean ± SD for triplicates in each group.
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occur because of the induction of T cell apoptosis in normal T cells and that immune cells in B6/lpr mice highly expressing FasL induce Fas-mediated and rapid apoptosis of T cells.

Discussion

In this study, we confirmed that normal T cells failed to survive in a Fas-deficient immune condition using the transfer experiments. In addition, the homeostatic proliferation of T cells in lymphopenic recipients of lpr mice and Ag-specific T cell response in lpr mice were not observed. These findings are consistent with the results in the previous reports regarding the failure of normal lymphocyte survival in lpr hosts (17, 18). The phenomenon was thought to occur because of the induction of T cell apoptosis in lpr recipients because the transferred T cells did not migrate in any specific organs other than lymphoid tissues and the liver.

Because the diminishment of normal T cells was observed in PBMCs in 30 min immediately after the transfer, rapid death of transferred T cells may have occurred in lpr recipients. However, T cell diminishment in lpr donor mice was undetectable. Thus, the rapid T cell death occurred by the presence or absence of the Fas molecule on these cells.

Although enhanced FasL expression on immune cells in lpr mice has been described previously (18, 20), it was unclear as to which subset of immune cells in lpr mice overexpressed FasL molecule. In this study, mRNA expression of FasL of all subsets in the spleen and PBMCs of lpr mice was significantly higher than that of control mice. In particular, FasL mRNA expression in CD11b+ macrophages in lpr mice was significantly increased compared with that in control mice. In addition, when thioglycolate-elicited PECs, including a number of macrophages, were used to analyze the interaction with T cell in vivo and in vitro, enhanced rapid death and clearance of T cells by lpr macrophages was observed. Furthermore, phagocytic activity of lpr macrophages was considerably enhanced compared with control macrophages. These results suggest that lpr macrophages can phagocyte apoptotic T cells promptly as well as induce the rapid T cell death in the periphery. The lpr macrophages with enhanced expression of FasL may induce rapid death of T cells and promptly engulf the apoptotic cells by FasL-independent phagocytosis. There may be unknown cellular mechanism like “Eat me signal.”

FasL, a type II transmembrane protein belonging to the TNF superfamily, is a well-characterized apoptosis initiating protein (32–34). Some transcription factors have been shown to regulate FasL gene expression, including specificity protein-1, IFN regulatory factor-1, NF in activated T cells and NF-kB (35–37). NF-kB plays key roles in differentiation and activation of macrophages (22, 38). Our results suggest that the direct contribution of macrophages to the induction of rapid death of T cells is very important for effective phagocytosis of apoptotic T cells. Furthermore, our results imply that the induction of expression of FasL in macrophages by NF-kB is negatively controlled by Fas signaling. This is related to our previous report that Fas signaling controls RANKL signaling following NF-kB activation in dendritic cells (39). Fas signaling may play important roles in NF-kB activation in relation to cell activation, survival, and growth in addition to apoptosis.

As to the relationship between FasL expression and phagocytosis in macrophages, it was reported that enhanced expression of FasL on Kupffer cells is associated with phagocytosis of apoptotic T cells in human liver allografts (40). Although further experiments will be needed to confirm the cellular mechanism, FasL-enhanced macrophages may engulf apoptotic cells effectively.

It is widely established that Fas expression on peripheral T cells plays a key role in AICD to maintain peripheral immune system (2, 3, 5). Fas expression on T cells increases by TCR signaling (41). In addition, some cytokines such as IL-2 and/or IFN-γ trigger the enhancement of Fas expression on T cells (42). Our results imply that an extremely high concentration of IFN-γ in the serum of lpr mice acts on the induction of Fas expression on the transferred T cells directly. When T cells from IFN-γR knockout mice were transferred into lpr mice, the rapid death and diminishment of T cells was not observed. These results strongly suggest that Fas expression on peripheral T cells is controlled through the IFN-γ/IFN-γR.

With regard to the control of the Fas expression of cells other than the T cell, it was reported that TNF-α can control the expression of fibroblasts in addition to IFN-γ (30). Although we found a high concentration of IFN-γ in the serum from lpr mice, the level of TNF-α concentration in the serum from lpr mice was similar to that from control mice (data not shown). In this study, when naive T cells from normal mice were transferred, they were not activated and slightly expressed the Fas molecule on the cell surface. Because of the exposure to the high concentration of IFN-γ in lpr mice, Fas expression on T cells rapidly increased. The immune cells highly expressing FasL, including macrophages of lpr mice may induce the rapid apoptosis of T cells, and the macrophages in the peripheral immune system may rapidly phagocytize the apoptotic T cells.

In contrast, many reports indicate that B cells are maintained by B and Bim-dependent apoptosis to protect autoimmunity (43–45). In our experiment, CD19+ B cells failed to undergo apoptosis in Fas-deficient host, although Fasl expression on immune cells was enhanced. This finding implies that rapid T cell death may be triggered by cell–cell contact between normal T cells and Fas-deficient macrophages through the interaction with cell surface molecules such as MHC class II, costimulatory molecules, or TCR, although its precise mechanism has been still clarified.

Our results suggest that Fas signaling contributes to nonapoptotic functions such as the phagocytic activity of macrophages. Fas promotes the differentiation, proliferation, and maturation in several cells (1, 2, 46). Fas-associated death domain–mediated activation of caspase 8 is essential for the process of apoptosis of various cells (12, 47). In addition, it was reported that Rho GTPase Rac1 sensitizes T cells to Fas-induced apoptosis correlated with Rac-mediated cytoskeletal reorganization, dephosphorylation of the ezrin/radixin/moesin family of cytoskeletal linker proteins, and the translocation of Fas to lipid raft microdomain (48). However, the molecular mechanism for controlling the phagocytic activity of macrophages through Fas signaling has yet to be elucidated.

Although it has been reported that normal immune cells failed to survive in lpr recipients, the precise mechanism for its phenomenon remained unclear. In this study, we found that abnormal macrophages in lpr mice play critical roles in the disorder of the peripheral immune system. Our findings are thought to be important for therapeutic strategies for immune disorders such as ALPS or the other autoimmune diseases related to the abnormal expression of Fas on immune cells. In addition, this study suggests that Fas expression on macrophages contributes to the survival of T cells in the peripheral immune system. Taken together, this study strongly suggests that Fas-expressing macrophages play a pivotal role in maintaining T cell homeostasis in addition to AICD in the periphery.
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Disclosures
The authors have no financial conflicts of interest.

References
**Supplementary figure legend 1:**
T cells (2 × 10^6) from B6 mice were labeled with XenoLight DiR and transferred into B6 and B6/lpr mice. The donor T cells in the liver and lungs of the recipients at 30 min after the transfer were detected using an *in vivo* imaging analyzer. Photos are representative of four mice in each recipient group.

**Supplementary figure legend 2:**
Phagocytic activity of CD11b+ macrophages in PECs was evaluated by uptake of FITC-conjugated beads *in vitro*. Cells fixed with 0.5% PFA were treated with 0.001% Triton X, and then washed in PBS. CD11b+ FITC+ cells were evaluated by flow cytometer. Results are shown as mean ± SD for five mice in each group. **p < 0.005.

**Supplementary figure legend 3:**
B cells (1 × 10^6) purified from spleen of GFP TG mice were i.p. injected into thioglicolate-treated B6 and B6/lpr mice. At 24 hours after the injection, GFP+ B220+ B cells in PECs were analyzed by flow cytometer. Results are shown as mean ± SD for three mice in each group.

**Supplementary figure legend 4:**
B6 T cells were stimulated for 12 hours with plate-bound anti-CD3 mAb (0.5 μg/ml) in the presence of recombinant IFNγ (0, 10, 25, and 100 ng/ml). Relative Fas expression to isotype control on T cells was shown as mean ± SD for triplicates. Mean fluorescence intensity: MFI *p < 0.005.
Supplementary Figure 1.
Supplementary Figure 2.
Supplementary Figure 3.
Supplementary Figure 4.