Fas Engagement Induces the Maturation of Dendritic Cells (DCs), the Release of Interleukin (IL)-1β, and the Production of Interferon γ in the Absence of IL-12 during DC–T Cell Cognate Interaction: A New Role for Fas Ligand in Inflammatory Responses

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Abstract

Ligation of the Fas (CD95) receptor leads to an apoptotic death signal in T cells, B cells, and macrophages. However, human CD34+–derived dendritic cells (DCs) and mouse DCs, regardless of their maturation state, are not susceptible to Fas-induced cell death. This resistance correlates with the constitutive expression of the Fas-associated death domain–like IL-1β–converting enzyme (FLICE)–inhibitory protein (FLIP) ligand. We demonstrate a new role of Fas in DC physiology. Engagement of Fas on immature DCs by Fas ligand (FasL) or by anti-Fas antibodies induces the phenotypical and functional maturation of primary DCs. Fas-activated DCs upregulate the expression of the major histocompatibility complex class II, B7, and DC–lysosome-associated membrane protein (DC-LAMP) molecules and secrete proinflammatory cytokines, in particular interleukin (IL)-1β and tumor necrosis factor α. Mature DCs, if exposed to FasL, produce even higher amounts of IL-1β. Importantly, it is possible to reduce the production of IL-1β and interferon (IFN)–γ during DC–T cell interaction by blocking the coupling of Fas–FasL with a Fas competitor. Finally, during cognate DC–T cell recognition, IL-12 (p70) could not be detected at early or late time points, indicating that Fas-induced, IFN-γ secretion is independent of IL-12.

Key words: dendritic cells • Fas • interleukin 1β • FLIP • interferon γ

Introduction

The first step in any adaptive immune response is the activation of naive T cells in the LN. Once stimulated by infection, dendritic cells (DCs) migrate to the draining LN where naive T cells circulate and can encounter their specific antigen on the DC surface (1). Because this is a rare event, the prolongation of cell survival and the correct activation state of DCs are of primary importance in order to control and sustain the immune response.

We have previously reported that DCs express the Fas receptor (CD95) (2), but its role on DCs is still a matter of debate (3, 4). Stimulation of the Fas receptor normally induces an apoptotic death signal. However, in T cells the interactions between Fas and Fas ligand (FasL) do not always induce a death signal (5). Indeed, depending on the state of T cell activation, Fas–FasL interaction can also lead to T cell activation and proliferation (6, 7). Thus, signaling through Fas in the early stages of an immune response might amplify the T cell response, whereas the signaling through Fas could downsize the response via apoptosis of T cells in later stages. Upon Fas activation and trimerization, a set of effector proteins is recruited to this receptor, forming
the death inducing signaling complex. Fas-associated death domain (FADD), the first protein that binds to Fas, recruits caspase-8, initiating a cascade of events resulting in efficient cell death (8). Fas-induced apoptosis can be blocked by FADD-like IL-1β–converting enzyme (FLICE)–inhibitory proteins (FLIPs) (9). Furthermore, FLIP mediates the activation of nuclear factor κB and extracellular signal–regulated kinase (Erk), diverting Fas-mediated death signals into signals that lead to proliferation and/or differentiation (10). Both of these signaling pathways are activated by LPS and play important roles in DC maturation and survival (11).

Recently, the deregulation of Fas–Fasl interactions has been considered as one of the major causes of exaggerated immune responses, such as organ-specific autoimmune diseases (12). In insulin-dependent diabetes mellitus, B islet cells express Fas on their surface in response to IL-1β and become susceptible to Fas-induced cell death by activated T cells (12).

In this study, we confirm that Fas is unable to induce DC death due to a constitutive FLIP expression and further demonstrate that Fasl triggers both a phenotypical and functional maturation of DCS, as well as the secretion of proinflammatory cytokines, in particular IL-1β and TNF-α.

Materials and Methods

Cells and Reagents. Purification of human CD34+ cells, primary culture with FLT3-L, thrombopoietin, and stem cell factor, and induction of DCS were performed as described previously (13). Two growth factor–dependent DC mice lines, D1 and D8, were used as well as bone marrow–derived fresh DCS (2, 14) and were grown as described previously (2, 14). Bone marrow cells were grown for 15–20 d and the homogeneity of the DC culture was evaluated by cytofluorimetry. CD4+ T cells were purified from TCR-OVA DO11.10 transgenic mice, bred in BALB/c background, by positive selection with an anti–CD4 antibody coupled to magnetic microbeads, using MiniMacS Separating columns (all from Miltenyi Biotec). LPS (Escherichia coli serotype 026:B6) was purchased from Sigma-Aldrich. FasL from Ortho Diagnostic Systems, anti–CD1a (clone OKT6) from Ortho Diagnostic Systems, anti–HLA-DR (clone L243) from Becton Dickinson, anti-CD80 (clone BB1) and anti-CD86 (clone IT2.2) from BD Pharmingen. Anti–DC-LAMP antibody (clone 510 F1.65.13) was a gift of Dr. S. Lebeque (Schering-Plough, Dardilly, France). Other reagents used were as follows: polyclonal mouse IgG reagent grade from Sigma-Aldrich and anti-CD34 mIgG-coated M450 Dynabeads from Dynal. LPS-matured or nontreated murine DCS were cultured either with anti-Flag Enhancer and Fasl, anti-CD95 (Jo-2), or an isotype-matched control, at the indicated concentrations. After activation, cells were incubated with one of the following mAbs from BD Pharmingen: anti–I-A/–I-Eγ (2G9), anti–CD86 (B7.2), anti–CD95 (Jo-2), or hamster IgG (G235–2356). Staining was carried out in the presence of 2–4G2 to block Fc receptor binding. For each sample, culture supernatants were collected for the cytokine production assay.

Cytokine Assays. TNF-α, IL-1β, IL-10, and IFN-γ were measured by using murine DuoSet™ following the manufacturer’s instructions (R&D Systems). IL-12 was tested using IL-12 capture (anti–IL-12 p70; 9A5) and detection (anti–IL-12 p40; 5C3). These antibodies and recombinant IL-12 were provided by Dr. D.H. Presky (Hoffman-La Roche, Nutley, NJ). IL-2 was tested by measuring [3H]thymidine incorporation of an IL-2–dependent CTL clone (CTLL).

Assessment of apoptosis. Apoptosis of DCS was assessed by flow cytometry. For murine cells, the exposure of phosphatidylserine residues on the cells’ surface was measured using FITC–conjugated annexin V (BD Pharmingen) and dead cells were identified with 1.25 μg/ml propidium iodide (Sigma-Aldrich). Alternatively, human DCS were incubated on ice in 300 ml of saline, 3% FCS containing 10 μg/ml of 7AAD (Sigma-Aldrich). Cells were analyzed using FL–3 for 7AAD staining, which identified living cells as 7AAD low, and dead and apoptotic cells as 7AAD high and medium, respectively (16).

Antigen Presentation Assay. D8 cells were seeded (106 cells/well) in flat-bottomed microtiter plates (Coming) pulsed with CD4+ T cells (5 × 104 cells/well) purified from TCR-OVA α/β transgenic mice (T/DC, 5:1) in the presence of decreasing concentrations of peptide OVA 327–339 as indicated, with or without 0.5 μg/ml of Fas-comp and in 0.3% normal mouse serum supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine (all from Sigma-Aldrich), and 50 μM 2-mercaptoethanol. Proliferative responses were assessed 48 h later by [3H]thymidine incorporation. Cell culture supernatants were collected at the indicated times for cytokine production measures.

Western Blot Analysis of FLIP. Human CD34+–derived DCS (5 × 106) were lysed in 50 μl of lysis buffer (1% NP-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and Complete™ protease inhibitor cocktail tablets [Boehringer]). 30 μg of protein per lane was subjected to electrophoresis under reducing conditions and transferred to nitrocellulose (Hybond ECL; Amersham Pharmacia Biotech). Blots were probed with anti-FLIP mAbs (Dave 2; Alexis), visualized using horseradish peroxidase–labeled, anti-rat Abs (Southern Biotechnology Associates, Inc.), and ECL blotting substrate (Amersham Pharmacia Biotech). Quantification on films was performed using the ONE D-Scan program (Scanalytics) as described previously (17).

Results

Fas Is Constitutively Expressed by DCS and Is Upregulated by LPS or TNF-α. Similar to mouse DCS (2), nonstimulated immature, human CD34+–derived DCS were found to express Fas (Fig. 1 A). Therefore, we studied the regulation of
Fas during maturation of human and mouse DCs induced by TNF-α or LPS. Incubation of human DCs with 40 ng/ml of TNF-α (Fig. 1 A) or LPS (data not shown) for 24 h induced a slight upregulation of Fas. We next incubated a well-defined, mouse spleen long-term growth factor-dependent DC line (D1 cells) or primary murine bone marrow–derived DCs (mBM-DCs) with LPS (10 μg/ml), which induced a significant upregulation of Fas that was more pronounced in primary mBM-DCs (compare Fig. 1, B and C). Interestingly, incubation of agonistic anti-Fas antibodies (Jo-2) resulted in a dose-dependent downregulation of Fas (not shown) at early time points and in the upregulation of Fas at late time points (not shown).

**DCs Are Resistant to Fas-induced Apoptotic Cell Death.** Since the induction of cell death by FasL remains controversial (3, 4, 18), we have studied the effects of FasL or the Jo-2 antibody on DCs. As shown in Fig. 2, A and B, both human and mouse DCs were resistant to Fas-induced cell death. Human CD34⁺–derived immature DCs or 72-h, TNF-matured DCs (data not shown) were treated with FasL and enhancer for 24 h and analyzed using 7AAD staining (see Materials and Methods). No significant increase of dead and apoptotic cells was noticed after FasL treatment for 24 h in immature DCs (Fig. 2 A) or TNF-matured DCs (data not shown). D1 cells and mBM-DCs, pretreated or not with LPS for 20 h, were incubated with different concentrations of anti-Fas antibody for 6 h. Cells were double-stained with FITC-conjugated annexin V and with propidium iodide. Cells single positive for annexin V were considered as undergoing early apoptosis. As shown in Fig. 2 B, regardless of the concentration of anti-Fas used, a maximum 10% of the D1 cells underwent apoptosis (when subtracting the amount of apoptotic cells after LPS treatment), and no apoptosis was observed when anti-Fas was tested on immature cells. Similar results were obtained with fresh mBM-DCs (not shown). Finally, no significant cell death occurred in mature or immature DCs during cognate DC–T cell interaction (not shown).

**DCs Constitutively Express FLIP.** Fas-induced apoptosis can be blocked during signal transduction by FLIPs (9). We observed by Western blot analysis that FLIP was constitutively expressed by human and mouse DCs. Further...
more, FLIP was upregulated approximately twofold at 24 h of DC incubation with FasL in human DCs (Fig. 2, C and D). A similar upregulation of FLIP was observed after FasL, TNF-α, or LPS treatment in either human or mouse DCs (data not shown).

Fas Induces Phenotypical Maturation of DCs. Subsequently, we examined the potential role of FasL in DC maturation (Fig. 3). Human (Fig. 3 A) and mouse (Fig. 3 B) DCs were incubated for 18–24 h with 0.1 μg/ml FasL and with 0.5 μg/ml of enhancer. This stimulus induced a strong upregulation of maturation markers such as MHC class II, CD40, CD80, CD83, and CD86 on the cell surface of human CD34+–derived DCs as well as of intracellular DC-LAMP (Fig. 3 A). The extent of maturation induced by FasL treatment was comparable to that induced by TNF-α or LPS (data not shown). Moreover, human and mouse DCs could be activated by FasL, but not by boiled FasL, excluding a possible endotoxin contamination. The effect of FasL was reproduced by the Jo-2 antibody. As shown in Fig. 3 C, as low as 0.01 μg/ml of Jo-2 antibody was sufficient to induce the maturation of both primary mBM-DCs and the D1 cell line, whereas an isotype control for the Jo-2 antibody had no effect (Fig. 3 C).

Fas Induces Functional Activation of Immature and Mature DCs. Cytokine secretion by DCs was tested in culture supernatants after Fas engagement and was compared with that induced by LPS (Fig. 4). Although the amount of TNF-α produced was comparable among LPS- and Fas-matured DCs (Fig. 4 A), Fas-matured DCs secreted more IL-1β (Fig. 4 B). If LPS-matured DCs were subsequently exposed to anti-Fas antibody, the production of IL-1β greatly increased (600 vs. 20 pg/ml with LPS alone, Fig. 4 B) as well as the amount of secreted IL-10 (Fig. 4 C). In contrast, the amount of bioactive IL-12 (p70) secreted was very low in Fas-matured DCs (Fig. 4 D). When we analyzed fresh mBM-DCs, similar results were obtained (Fig. 5, A and B).

Blocking Fas–FasL Interaction during Cognate DC–T Cell Interaction Partly Inhibits the Production of IL-1β and IFN-γ. To gain insight on the functional activation of DCs by FasL, we measured cytokine production by DCs during antigen-specific T cell proliferation in the presence or ab-
ence of a Fas-comp (15). CD4+ T cells were purified from TCR-OVA DO11.10 mice and incubated with a homogenous mouse bone marrow–derived, long-term DC line (D8; T/DC, 5:1) in the presence of varying concentrations of OVA 327–339 peptide, with or without 0.5 μg/ml Fas-comp. This concentration of competitor completely inhibited Fas-induced activation of DCs (not shown). As shown in Fig. 6 A, T cell proliferation was not reduced in the presence of Fas-comp; rather, an increase in thymidine uptake was observed in the presence of Fas-comp, probably because of a direct interaction with FasL on T cells. Consistently, the amount of IL-2 produced increased in the presence of Fas-comp. In contrast, DCs were impaired in their capacity to secrete IL-1β, as shown by the inhibition of cytokine released 48 h after DC–T cell interaction in the presence of Fas-comp (Fig. 6 C). Although the production of IL-2 by T cells was increased in the presence of Fas-comp, the amount of IFN-γ produced was reduced (Fig. 6 D), suggesting an important role of Fas–FasL interaction for the polarization of T cells. The reduced inhibition of IFN-γ observed at 48 h of antigen presentation could result from a suboptimal amount of Fas-comp when T cells are actively proliferating. Interestingly, the production of IFN-γ was not dependent on IL-12 secretion because bioactive IL-12 (p70) could not be detected in culture supernatants throughout the entire proliferative response (Fig. 6 E).

**Discussion**

Unlike B cells and macrophages, which are susceptible to Fas-induced cell death (19–21), DCs are largely resistant to FasL. Although it has been reported that murine DCs undergo apoptosis after cognate T cell interaction, partly mediated by Fas–FasL interaction (4), several studies have shown that, in vivo, T cells induce survival of DCs (22), and that in vitro, neither T cells nor FasL induce DC death (3, 18). In this report, we confirm that DCs are resistant to
secretion of proinflammatory cytokines such as TNF-
DCs drives their phenotypical maturation and induces the
we demonstrate that the engagement of Fas on immature
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stitution in DCs of FLIP, an intracellular inhibitor of apoptotsis.
Fas-mediated cell death, irrespective of their maturation
stage. This resistance correlates with the constitutive expres-
sion in DCs of FLIP, an intracellular inhibitor of apoptosis.

DCs have the unique capacity to prime T cells, and the
costimulatory surface molecules B7 and CD40 contribute to
this function (1). The counterpart of B7 on T cells, CD28 for instance, increases the duration of TCR signal-
ing which correlates with T cell activation (23). A nonca-
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The engagement of Fas on naive T cells, in suboptimal
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naive T cells into functional T cells (5, 10). In this report,
we demonstrate that the engagement of Fas on immature
DCs drives their phenotypical maturation and induces the
secretion of proinflammatory cytokines such as TNF-α and
IL-1β. Moreover, mature DCs exposed to anti-Fas pro-
duce increased amounts of IL-1β, indicating a different
outcome of Fas engagement of immature and mature DCs.
IL-1β was also produced during cognate DC–T cell inter-
action and its secretion was blocked with Fas-comp, indi-
cating a direct in-volvement of Fas–FasL for its production.
Since FLIP is constitutively expressed in DCs, and as a con-
sequence, caspase-8 and IL-1β–converting enzyme are in-
hibited, this IL-1β production is likely to be caspase-1 in-
dependent. This result concurs with recent data showing that FasL induces caspase-1–independent IL-1β secretion in peritoneal exudate cells (26).

Several reports have shown an important role of IL-1β in the pathogenesis of organ-specific immunity. The expo-
sure of β islet cells to IL-1β induces a selective and func-
tional expression of Fas on β islet cells through the produc-
tion of nitrous oxide (27). Subsequently, Fas expressed on
β islet cells is recognized by FasL on activated T cells caus-
ing tissue destruction. Since mature DCs produce large
amounts of IL-1β in response to Fas–FasL interaction, we
can speculate that during a chronic inflammatory state, such
as in the case of organ-specific autoimmunity, mature DCs
recruited at the inflammation site are activated by FasL-
expressing T cells to produce IL-1β, which in turn can con-
tribute to the progression of the disease. A recent report, in
agreement with this hypothesis, shows that transgenic non-
obese diabetic mice expressing FasL in β cells are more
sensitive to diabetogenic T cells (28) in a FasL-dependant
manner. Since transgenic β cells were also expressing higher amounts of Fas, it is possible that these cells, by ex-
pressing FasL, were directly activating DCs for IL-1β pro-
duction, which in turn would be responsible for Fas induc-
Accordingly, transplantation of the fetal pancreas from
transgenic mice expressing murine CD95L on their β islet
cells under the kidney capsules of allogeneic animals re-
sulted in rapid graft rejection (29).

Though murine myeloid DCs did not secrete IL-12 after Fas ligation, T cells did produce IFN-γ in a Fas–FasL-
dependant manner. A Fas-comp did not affect T cell prolif-
eration and IL-2 production. Rather, an increased prolif-
eration was observed, suggesting that Fas expressed on DCs
may have a dual role as both the activator of T cell prolif-
eration and the director of T cell polarization into a Th1
phenotype, even in the absence of IL-12. Further exper-
iments on the effects of Fas–FasL interaction on T cells will
be needed to address this question.

![Figure 6. IL-1β and IFN-γ release is partly inhibited by Fas-comp during cognate DC-T cell interaction. (A) DO11.10 Tg T cell proliferative response to the OVA 327–339 peptide in the presence (∗) or absence of 0.5 µg/ml Fas-comp (●). T cells were incubated with or without D8 cells, and T cell proliferation was measured by [3H]thymidine incorporation. (B) IL-2 level in culture supernatants from the experiment in A at 5 µg/ml of peptide with Fas-comp (Fas-comp, gray bars) or without Fas-comp (ctrl, white bars) (0.5 µg/ml). (C) IL-1β was greatly reduced in the presence of Fas-comp at 24 h, and little cytokine production was ob-
served in the absence of DCs (CD4+T). (D) IL-12 was almost undetectable (<5 pg/ml) at all analyzed time points, both with
and without Fas-comp. Results are representative of three inde-
pendent experiments.](image-url)
Finally, our results demonstrate that Fas–FasL interaction induces the maturation of DCs, the secretion of large amounts of IL-1β by DCs, and drives the polarization of T cells into a Th1 phenotype. These findings explain, at least in part, the adverse effects obtained by the transgenic expression of FasL in transplanted organs (28, 29) and puts clear constraints on the therapeutic use of FasL in autoimmune diseases.

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