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Phagocytosis, a Potential Mechanism for Myeloid-Derived Suppressor Cell Regulation of CD8+ T Cell Function Mediated through Programmed Cell Death-1 and Programmed Cell Death-1 Ligand Interaction

Young-June Kim,* Su-Jung Park,†‡ and Hal E. Broxmeyer*

CD8+ T cells become exhausted, inducing cell surface protein programmed cell death-1 (PD-1) as chronic virus diseases or tumors progress, but underlying mechanisms of this are unclear. We previously showed that M-CSF is important for developing tolerogenic dendritic cells (DCs) from human CD14+ monocytes. In this article, we identify M-CSF–derived DCs (M-DCs) after stimulation with IL-10 as myeloid-derived suppressor cells with additional tolerogenic activities to CD8+ T cells. IL-10 increased PD-1 ligand expression on M-DC, and IL-10–stimulated M-DCs (M-DC/IL-10) induced expression of PD-1 on, and apoptosis of, CD8+ T cells and phagocytosed CD8+ T cells. Enhanced phagocytic activity of M-DC/IL-10 required IFN-γ, which further increased PD-1 ligand and PD-2 ligand expression on M-DC/IL-10. IFN-γ-stimulated M-DC/IL-10 cells were phenotypically macrophage-like cells with little or no expression of CD86, a costimulatory molecule, but with high expression levels of CD14, CD200R, and CD80. No phagocytic activity was detected with GM-CSF–derived DCs. We propose that phagocytosis by IFN-γ-stimulated M-DCs may serve as a myeloid M-DC-like cell that phagocytoses CD8+ T cells and induces programmed cell death-1 (PD-1) expression, which may be a mechanism by which IFN-γ–producing CD8+ T cells are tolerized after type 1 immune responses to chronic virus or tumor, and that IFN-γ links effector CD8+ T cells to their phagocytic clearance.

IL-10 is a potent immunosuppressive cytokine that inhibits the ability of DCs to mature into functional APCs that have low-level secretion of proinflammatory cytokines and expression of costimulatory receptors (25, 32). IL-10 is often increased in persistent infections in mice and humans (15, 33), and is implicated in impaired T cell response to chronic viral infections (32–34). Consistent with this, IL-10R blockade increases proliferative capabilities of HIV- and HCV-specific T cells (35, 36). IL-10 upregulates PD-L1 expression on peripheral blood CD14+ monocytes (15, 37), and PD-L1 upregulates IL-10 production (32, 38) as part of an immunosuppressive circuit. IL-10 and PD-L1 may cooperate to promote exhaustion of CD8+ T cells probably in synergy during persistent viral infections (15, 32, 39). Thus, IL-10 may be involved in switching functional properties of immunogenic DCs to tolerogenic DCs through mediation of PD-L1 signaling.

Although PD-1/PD-L1 signaling, IL-10, and MDSCs are important central immune suppressive mediators, the mechanisms by which the suppressive signaling pathways merge to execute exhaustion processes, particularly in CD8+ T cells, remain elusive. We previously demonstrated that M-CSF–derived DCs (M-DCs) were tolerogenic to CD4+ T cells (40), Unlike GM-CSF–derived DCs (GM-DCs), M-DCs produce high levels of IL-10, but not IL-12. IL-10–producing MDSCs may contribute to an increase of green fluorescence in M-DCs. In some cases, CD8+ T cells were cultured in IL-15 (20 ng/ml) for 5 d with or without dexamethasone (10−7 M) or anti-CD3 coated to culture plates at 0.1 μg/ml. DCs were mixed with allogeneic CD8+ T cells pre-incubated for 5 d in IL-15 and cultured for an additional 4 d in 20 ng/ml IL-15, 20 ng/ml M-CSF, and 20 ng/ml IL-10 in complete RPMI in the absence or presence of 50 ng/ml IFN-γ. CD8+ T cells were stained with CFSE (10 M) in PBS before being mixed with DCs. Cells in cocultures were examined under the microscope or harvested for flow cytometric analysis for expression of surface markers. In some cases, cell cultures were carried out on glass slides for observation under confocal microscopy.

Flow cytometric analysis

Harvested cells were washed with PBS supplemented with 1% BSA. Fcs on cells were pregel coated with phosphatidyl glycerol (Sigma-Aldrich) on ice for 15 min. Cells were stained for 30 min at 4°C with the following FITC-conjugated Abs: anti-CD14 (BD Pharmingen, San Diego, CA); PE-conjugated Abs: CD-1 (105 and MIH4; eBioscience), PD-L1 (MIH1; eBioscience); PE-Cy7–conjugated Ab: PD-L1 (MIH1; eBioscience), PE–Cy5–conjugated CD86 (BD Pharmingen, CA); and PE-Cy5–conjugated Ab: CD11c (BD Pharmingen, CA), CD1–(MIH4; BD Pharmingen, CA), or isotype controls (eBioscience). CD8+ T cells and DCs in coculture were analyzed using gating process for CD8+ and CD11c+ cells, respectively. CFSE-stained CD8+ T cells and fluorescence in DCs were analyzed at channel 1 (FL1). Apoptosis of CD8+ T cells after culture with or without M-DC was measured by staining with Annexin V-PE. 

Quantification of CD8+ T cells phagocytosed by M-DC/IL-10

CD8+ T cells were cultured in IL-15 (10 M) in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT) containing 2 mM L-glutamine, 55 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin and 20 ng/ml IL-4 for 5 d in the presence of either recombiant human M-CSF (20 ng/ml) or 400 U/ml recombinant human GM-CSF to respectively generate M-DCs and GM-DCs. A total of 20 ng/ml IL-10 was included in M-DC and GM-DC generation cultures to produce IL-10–stimulated M-DCs (M-DC/IL-10) and IL-10–stimulated GM-DCs (GM-DC/IL-10), respectively. In some cases, 50 ng/ml TNF-α (PeproTech) was added to the M-DC/IL-10 generation cultures. Half of culture medium was exchanged every 3 d, unless otherwise indicated. M-DC/IL-10 and GM-DC/IL-10 were further cultured for an additional 4 d with or without IFN-γ at 50 ng/ml to generate IFN-γ–stimulated M-DC/IL-10 (M-DC/IL-10/IFN-γ) and IFN-γ–stimulated GM-DC/IL-10 (GM-DC/IL-10/IFN-γ), respectively.

Coculture of DCs and CD8+ T cells

DCs harvested on day 5 were placed in 96-well flat-bottom plates (1 × 104 cells in 100 μl medium; Costar, Corning, NY). Before coculture with DCs, CD8+ or CD4+ T cells (1 × 106 cells/ml) were incubated for 5 d in complete RPMI 1640 medium supplemented with 10 ng/ml IL-15. IL-2 (100 U/ml) was added to CD4+ T cell cultures in addition to IL-15 to maintain cell viability. In some cases, CD8+ T cells were cultured in IL-15 (20 ng/ml) for 5 d with or without dexamethasone (10−7 M) or anti-CD3 coated to culture plates at 0.1 μg/ml. DCs were mixed with allogeneic CD8+ T cells pre-incubated for 5 d in IL-15 and cultured for an additional 4 d in 20 ng/ml IL-15, 20 ng/ml M-CSF, and 20 ng/ml IL-10 in complete RPMI in the absence or presence of 50 ng/ml IFN-γ. CD8+ T cells were stained with CFSE (10 M) in PBS before being mixed with DCs. Cells in cocultures were examined under the microscope or harvested for flow cytometric analysis for expression of surface markers. In some cases, cell cultures were carried out on glass slides for observation under confocal microscopy.

Confocroscopy

DCs cocultured with T cells for 2–4 d were allowed to adhere to glass slides (LabTek Chamber Slide; Nunc, Rochester, NY), washed with PBS to remove loosely bound T cells, and then fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were then stained with DAPI and mounted with ProLong Gold antifade reagent (Invitrogen, Eugene, OR). Fluorescence analysis of cells was performed using the Zeiss LSM 510 confocal laser scanning system (Carl Zeiss, Heidelberg, Germany) at 100× magnification.

Statistical analysis

A two-tailed paired Student t test (unless otherwise indicated) was used to determine statistical significance. The p values <0.05 were considered significant.
Results

DCs derived by a combination of M-CSF and IL-10 increase PD-1 expression on CD8+ T cells on coculture

We previously demonstrated that M-DCs showed tolerogenic potential to allogeneic CD4+ T cell proliferation. M-DCs were characterized by their ability to secrete IL-10 at high levels but not IL-12, on LPS stimulation (40). However, whether M-DCs act as suppressor cells to CD8+ T cell functions has not been examined. PD-1 is involved in suppressing CD8+ T cell response to chronic infection and tumor (5, 42). We were interested in determining whether M-DCs affect PD-1 expression on CD8+ T cells, because no specific type of APCs or DC subsets have been identified as being responsible for inducing PD-1 expression on CD8+ T cells.

Although IL-10 is induced during chronic infection and has been reported to be a potential factor for PD-1-mediated viral persistence, there is no clear understanding of the specific cells responsible for inducing PD-1 on CD8+ T cells.

To determine potential effects of M-DCs and IL-10-treated M-DCs on PD-1 induction on CD8+ T cells, M-DCs were generated in vitro by culturing human peripheral blood CD14+ monocytes with M-CSF and IL-4 in the absence and presence of IL-10 for 5 d. These cells, referred to as M-DC and M-DC/IL-10, respectively, were cocultured with allogeneic CD8+ T cells. Before coculture, allogeneic CD8+ T cells had been incubated for 5 d in IL-15 to simulate exhaustion processes of effector or memory CD8+ T cells. After coculture for 4 d, PD-1 expression levels on CD8+ T cells were measured. Typically, <10% of CD8+ T cells expressed PD-1 after a 5-d culture in IL-15. PD-1 was expressed on CD4+ T cells after a 5-d culture in IL-15 and IL-2, but their expression levels were lower compared with those on CD8+ T cells (<5%). M-DC/IL-10 increased PD-1 levels on CD8+ T cells to >20% as a result of coculture (Fig. 1A, 1B). This was in contrast with M-DC, which had little effect on PD-1 expression on CD8+ T cells. GM-DCs and GM-DC/IL-10 generated in the same way as M-DCs or M-DC/IL-10, but with GM-CSF instead of M-CSF, did not enhance PD-1 expression on CD8+ T cells (data not shown).

We then determined whether the presence of IFN-γ and IL-4, type 1 and 2 cytokines, respectively, and TNF-α, a proinflammatory cytokine, influence M-DC/IL-10 effects on PD-1 expression of cocultured CD8+ T cells.

FIGURE 1. M-DC/IL-10 possess a unique ability to induce PD-1 expression on CD8+ T cells, and IFN-γ enhances effect of M-DC/IL-10. A, CD8+ T cells were cultured for 5 d in the presence of IL-15 and cocultured in IL-15 for 4 d with DCs generated from peripheral CD14+ monocytes with M-CSF (M-DCs), M-CSF/IL-10 (M-DC/IL-10), M-CSF/IL-10/IFN-γ (M-DC/IL-10/IFN-γ), M-CSF/IL-10/IL-4 (M-DC/IL-10/IL-4), or M-CSF/IL-10/TNF-α (M-DC/IL-10/TNF-α) at a ratio of 1:1. Cells were stained for CD11c, CD8, and PD-1. Cells negative for CD11c were analyzed for PD-1 expression in CD8+ T cells. Percentage of PD-1–expressing CD8+ T cells are displayed in the second quadrant of dot plots. Dot plots are representative of results from four independent experiments. B, Bar histograms are expressed as mean percentage PD-1+CD8+ T cells ± SD from four independent experiments. C, CD8+ T cells were cultured for 4 d in the presence of IL-15 with conditioned media collected from corresponding DCs instead of by coculture with DCs. Percentage PD-1–expressing cells in a gated CD8+ T cell population was measured by flow cytometry and is displayed with SDs. Histograms are representative of averages of three independent experiments with each experiment carried out in triplicate.
IFN-γ enhanced M-DC/IL-10 effects on PD-1–expressing CD8+ T cells (Fig. 1A, 1B). Notably, presence of IL-4 or TNF-α during M-DC/IL-10 generation abrogated the enhancing effect of M-DC/IL-10 on percentage PD-1 expression. Our results suggest that M-DC/IL-10 may be an important mediator cell population limiting IFN-γ–producing CD8+ T cell responses. We next assessed whether increased percentage of PD-1 expression on CD8+ T cells by coculture with M-DC/IL-10 and M-DC/IL-10/IFN-γ was due to soluble factors secreted from these DCs. This was done by culturing CD8+ T cells with conditioned media collected from DC cultures at the 5th day of culture. Conditioned media from neither M-DC/IL-10 nor M-DC/IL-10/IFN-γ increased percentage of PD-1–expressing CD8+ T cells (data not shown). This suggests that contact with M-DC/IL-10 was responsible for induction of PD-1 expression on IFN-γ–producing CD8+ T cells. This indicates that IL-10 is important for inducing PD-1 on CD8+ T cells, but the effect is mediated through M-DC/IL-10.

IFN-γ induces PD-L1 expression at a high level on M-DC only after IL-10 stimulation

Because M-DC/IL-10 are unique in ability to enhance PD-1 expression on CD8+ T cells, we investigated whether M-DC/IL-10 in comparison with M-DC expressed PD-L1 (Fig. 2). DCs generally express costimulatory or coinhibitory molecules after DCs mature. We found that M-DC/IL-10 expressed higher levels of PD-L1 (34.5%) on the cell surface compared with M-DC (12.3%). PD-L1 expression levels were remarkably increased to 93.7% when M-DC/IL-10 were stimulated with IFN-γ for an additional 4 d. In contrast with M-DC/IL-10, M-DC failed to respond to IFN-γ as much as M-DC/IL-10, with only a slight increase in PD-L1 expression from 12.3 to 20.6%. GM-DC/IL-10/IFN-γ expressed a high level of PD-L1 expression (72.8%), but it was slightly lower than for M-DC/IL-10/IFN-γ. The results suggest that IFN-γ is a potent inducer of PD-L1, and IL-10 acts as a key mediator for a maximal response to IFN-γ.

M-DC/IL-10 are unique for low CD86 and high CD14 and CD200R expression

Because M-DC/IL-10 induced PD-1, a coinhibitory receptor on cocultured CD8+ T cells, we investigated whether M-DC/IL-10 differed from M-DC in expression of costimulatory receptors such as CD86, which is important for T cell activation. We found that M-DC/IL-10 expressed CD86 at much lower levels (26.1%) compared with M-DC (74.1%) (Fig. 3A). In contrast, M-DC/IL-10 maintained higher levels of CD14 (96.5%) than M-DC (47.6%). The expression levels of CD14 on M-DC/IL-10 levels are comparable with those on MΦs developed by M-CSF without IL-4 from CD14+ monocytes. IFN-γ reduced levels of CD86 expression on M-DC/IL-10 (3.9%), whereas maintaining high levels of CD14 expression. In contrast with IFN-γ, LPS, known to promote maturation of MΦs and DCs, increased expression of CD86 on M-DC/IL-10 (M-DC/IL-10/LPS) from 26.1 to 76.7%, reducing CD14 expression. This indicates that although both IFN-γ and LPS are considered to be proinflammatory, IFN-γ is unique in its inhibitory effects on CD86 expression on M-DC/IL-10. Therefore, a phenotype of M-DC/IL-10/IFN-γ with little expression of costimulatory CD86 but high expression of coinhibitory PD-L1, as shown in Fig. 2, may relate to their tolerogenic effect on CD8+ T cells. Like M-DC/IL-10, GM-DC/IL-10 expressed lower levels of CD86 (58.8%) compared with GM-DC (88.9%). Similar to M-DC/IL-10, GM-DC/IL-10 expressed higher levels of CD14 (85.9%) compared with GM-DC (19.2%), indicating that IL-10 is equally inhibitory for M-DC and GM-DC with regard to differentiating into a mature form of DC. In contrast with the inhibitory effect of IFN-γ on reducing CD86 expression on M-DC/IL-10, IFN-γ increased CD86 expression on GM-DC/IL-10. This suggests that IFN-γ may have dual functions as an immune suppressor for M-DC/IL-10, as well as an immune activator for GM-DC/IL-10.

High expression of CD14 on M-DC/IL-10 and M-DC/IL-10/IFN-γ cells implies that they may represent a unique subset of myeloid-derived DCs or MΦs. To better define M-DC/IL-10 and M-DC/IL-10/IFN-γ cells, we assessed expression of CD200R on M-DC/IL-10 and M-DC/IL-10/IFN-γ because CD200R is expressed almost exclusively by myeloid cells, including MΦs, and delivers an inhibitory signal to myeloid cells to suppress CD8+ T cell immune responses, and thus acts as an inhibitory receptor (43). Consistent with this, CD200 expression on malignant cells is correlated with poor prognosis (44). Thus, we compared expression levels of CD200R on M-DC, M-DC/IL-10, and M-DC/IL-10/IFN-γ with that on prototype MΦs and conventional DCs (referred to as GM-DC in this article) developed in vitro by M-CSF and GM-CSF plus IL-4, respectively. Modest levels of CD200R were detected on MΦs and GM-DCs at 16.3 and 15.2%, respectively, when estimated by MFI (Fig. 3B). Relatively higher levels of CD200R were expressed on M-DCs (at 36.3%) compared with MΦs and GM-DC. Even higher levels of CD200R expression were observed in M-DC/IL-10 and M-DC/IL-10/IFN-γ (at 78.8 and 84.7%, respectively). Because CD200R is involved in tolerogenic activities by specific subsets of MΦs and DCs (45, 46), high expression of CD200R on M-DC/IL-10 and especially on M-DC/IL-10/IFN-γ may relate to the tolerogenic nature of these.
There are additional ligands for PD-1, such as PD-L2 and CD80 (4). Although PD-L2 and CD80 were expressed only at marginal levels, 7.7 and 18.7%, respectively, on M-DC/IL-10, they were increased to 34.6 and 70.3%, respectively, on M-DC/IL-10/IFN-γ, as shown in Fig. 3C, indicating that IFN-γ acts as an important factor for inducing PD-L2 and CD80, as well as PD-L1.

Viable CD8+ T cell numbers are decreased in the presence of M-DC/IL-10/IFN-γ

Because M-DC/IL-10 are intrinsically different from M-DC in high expression of cell surface PD-L1 and CD200R and in ability to induce PD-L1 expression on CD8+ T cells, we evaluated whether M-DC/IL-10 have a greater negative impact on survival of CD8+ T cells compared with M-DC, especially in response to IFN-γ. To test this possibility, numbers of viable CD8+ T cells were counted by flow cytometry, using gating for CD8+ T cells after 4-d coculture with M-DC, M-DC/IL-10, and M-DC/IL-10/IFN-γ (Fig. 4A). CD8+ T cells cocultured with M-DC/IL-10 were lower in numbers than those cocultured with M-DC. CD8+ T cell numbers were further reduced when they were instead cocultured with M-DC/IL-10/IFN-γ. Numbers of viable CD8+ T cells cocultured with M-DC/IL-10 or M-DC/IL-10/IFN-γ were below numbers of CD8+ T cells cultured with or without M-DC. In contrast with M-DC/IL-10, GM-DC/IL-10/IFN-γ had a minimal effect on CD8+ T cell numbers. M-DC or IFN-γ itself did not result in decreased CD8+ T cells (data not shown). We also evaluated the status of CD8+ T cells cocultured without or with M-DC, M-DC/IL-10, or M-DC/IL-10/IFN-γ by plotting the cells against forward scatter (FSC) and side scatter (SSC). The dot plots allowed us to distinguish viable CD8+ T cells from dead cells and DCs (Fig. 4B). Significant decreases of CD8+ T cell numbers with apparent dead cells were noticed after coculture with M-DC/IL-10. Even more cell loss was observed in CD8+ T cells cocultured with M-DC/IL-10/IFN-γ. The ability of M-DC/IL-10/IFN-γ to cause CD8+ T cell apoptosis

**FIGURE 3.** M-DC/IL-10/IFN-γ cells are characterized by low CD86 and high CD14 and CD200R expression. A, M-DC, M-DC/IL-10, M-DC/IL-10 stimulated without or with IFN-γ (M-DC/IL-10/IFN-γ) or LPS (M-DC/IL-10/LPS), and counterpart GM-DCs were compared for expression of CD86 and CD14 using anti–CD86-PE-Cy5 and anti–CD14-FITC. MΦ denotes a prototype MΦ developed with M-CSF in the absence of IL-4. Dot plots are representatives of six independent experiments. Numbers in dot plots represent percentage of CD86+ and percentage of CD14+ cells. B, Expression levels of CD200R on a prototype MΦ, GM-DC, M-DC, M-DC/IL-10, and M-DC/IL-10/IFN-γ cells were displayed as MFIs in histograms. Open curves in each histogram represent CD200R+ cells; shaded curves represent isotype controls. Flow cytometry histograms are representative of at least four independent experiments. C, M-DC/IL-10 and M-DC/IL-10/IFN-γ cells were compared for the expression of PD-L2 and CD80 using anti–PD-L2-PE and anti–CD80-PE. Open curves in each histogram represent PD-L2+ and CD80+ cells; shaded curves represent isotype controls. Flow cytometry histograms are representative of four independent experiments.
**FIGURE 4.** Viable CD8+ T cell numbers are decreased in the presence of M-DC/IL-10/IFN-γ. A, CD8+ T cells were cocultured without or with M-DC, M-DC/IL-10, M-DC/IL-10/IFN-γ, or GM-DC/IL-10/IFN-γ at a ratio of 1:1 for 4 d. The T cells were stained with anti-CD8-PE-Cy5.5 and anti-CD11c-allophycocyanin, and analyzed for the numbers of viable CD8+ cells by flow cytometry. B, CD8+ T cells cocultured without or with M-DC, M-DC/IL-10, or M-DC/IL-10/IFN-γ as described earlier were displayed in dot plots against FSC and SSC to identify viable CD8+ T cells (cells in circles) and DCs (cells in boxes), which are distinguished from dead cells (cells with low FSC and high SSC). Numbers depicted in the plots represent counts of viable CD8+ T cells, as representative results of six independent experiments. C, Apoptotic status of the cocultured CD8+ T cell populations was assessed by Annexin V staining. CD8+ T cells were dyed with CFSE before coculture, and CD8+ T cells were identified as CFSEbright cells after gating based on FSC and SSC, as shown in B. The numbers depicted in the plots represent percentage Annexin V+ cells in the CFSEbright CD8+ T cell populations, as representative results of four experiments.

A.

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B.

C.

was further substantiated by measuring Annexin V+CD8+ T cells after their coculture with M-DC/IL-10/IFN-γ (Fig. 4C). CD8+ T cells were dyed with CFSE before coculture to use CFSE as a marker for CD8+ T cells to avoid surface markers staining steps after coculture, which might affect the following Annexin V staining. CD8+ T cells cocultured without or with M-DC showed 8.3 and 9.6% AnnexinV, respectively. However, increased AnnexinV cells were found in CD8+ T cells (16.4%) when cocultured with M-DC/IL-10, and to even higher levels (23.7%) with M-DC/IL-10/IFN-γ. This suggests that M-DC/IL-10 induces apoptosis of CD8+ T cells, and this effect is more severe in the presence of IFN-γ.

**Microscopic images indicate phagocytic activity by M-DC/IL-10/IFN-γ**

M-DC, M-DC/IL-10, and M-DC/IL-10/IFN-γ are morphologically distinct, as shown in Fig. 5A. M-DC/IL-10 are larger and irregular in their shape with many granules in the cytoplasm compared with M-DC. M-DC/IL-10/IFN-γ showed even greater morphological changes expressing more granules with many thin filaments extending from the cell surface. We noticed numerous vacuoles in the M-DC/IL-10/IFN-γ cells when they were cocultured with CD8+ T cells under contrast microscopic observations (indicated by arrows in Fig. 5B). Interestingly, a large portion of the vacuoles were occupied by small, round cells, most likely CD8+ T cells. Such phenomena were rarely detected with M-DC/IL-10. Interestingly, a large portion of the vacuoles were occupied by CD8+ T cells. These observations suggest possible phagocytic activity of M-DC/IL-10/IFN-γ.

The many vacuoles occupied by CD8+ T cell-like cells in M-DC/IL-10/IFN-γ cells led us to speculate that loss of the CD8+ T cells as noted in Fig. 4A might result from phagocytic events. To verify this possibility, we cocultured CFSE-stained CD8+ T cells with M-DC, M-DC/IL-10, or M-DC/IL-10/IFN-γ for 4 d on glass slides. Before confocal microscopic observations, we removed loosely bound CD8+ T cells from adherent DCs by washing the glass slides with PBS. After cell fixation, staining with DAPI, and mounting on glass plates, we assessed the possibility that DCs acquired fluorescence because of engulfment of CFSE-stained CD8+ T cells under confocal microscopy (Fig. 5C). CD8+ T cells were distinguished from DCs by their green fluorescence, shape, and size. Indeed, we detected many M-DC/IL-10/IFN-γ cells fluorescent with speckles, presumably originating from CFSE-stained CD8+ T cells. M-DC/IL-10/IFN-γ became even more fluorescent than M-DC/IL-10 on coculture with CFSE-stained CD8+ T cells. We also noticed that many more CD8+ T cells remained associated with M-DC/IL-10/IFN-γ after washing, compared with those cultured with M-DC and M-DC/IL-10. This suggests that M-DC/IL-10/IFN-γ had stronger interactions with CD8+ T cells compared with M-DC and M-DC/IL-10. Importantly, addition of neutralizing anti–PD-L1 to the cocultures inhibited the detection of fluorescence in M-DC/IL-10/IFN-γ, as well as numbers of CD8+ T cells associated with M-DC/IL-10/IFN-γ (Fig. 5C). Based on these results, we suggest that M-DC/IL-10/IFN-γ are able to phagocytose CD8+ T cells, and that PD-L1 induced by IFN-γ may be involved, at least in part, in this phagocytic activity. These phenomena were unique for M-DC/IL-
IL-10 cocultured with CFSE-stained CD8+ T cells (6.5 and 21.9%; respectively; Fig. 6A). Neutralizing anti–PD-L1 also reduced CFSE staining CD8+ T cells (42.9%). Controls represent M-DC cultured with CFSE-stained CD8+ T cells without M-DC. The glass plates were washed with PBS to remove CD8+ T cells loosely bound to adherent DCs. The remaining cells were fixed with 4% paraformaldehyde for 10 min at room temperature, stained with DAPI, mounted, and observed under confocal microscope to detect fluorescence in DCs (original magnification ×400). Small, bright green, round cells represent CFSE-stained CD8+ T cells.

FIGURE 5. M-DC/IL-10/INF-γ cells show signs of phagocytic activity. A, Images of M-DC, M-DC/IL-10, and M-DC/IL-10/INF-γ were observed under contrast light microscopy (original magnification ×200). M-DC/IL-10/INF-γ were unique with many granules and filaments. B, M-DC/IL-10 and M-DC/IL-10/INF-γ were cocultured with the CD8+ T cells for 4 d. Cells were observed under contrast light microscope (original magnification ×200). Arrows indicate apparent vacuoles on the surface of M-DC/IL-10/INF-γ. Some of the vacuoles are occupied by small, round cells, presumably CD8+ T cells. C, M-DC, M-DC/IL-10, and M-DC/IL-10/INF-γ were cocultured with CFSE-stained CD8+ T cells on glass slides for 4 d without or with neutralizing anti–PD-L1. Control slide contained CD8+ T cells without M-DC. The glass plates were washed with PBS to remove CD8+ T cells loosely bound to adherent DCs. The remaining cells were fixed with 4% paraformaldehyde for 10 min at room temperature, stained with DAPI, mounted, and observed under confocal microscope to detect fluorescence in DCs (original magnification ×400). Small, bright green, round cells represent CFSE-stained CD8+ T cells.

Effects of apoptotic and activation status of CD8+ T cells on their responsiveness to phagocytosis, and estimated number of CD8+ T cells phagocytosed by M-DC/IL-10/INF-γ cells

To examine whether apoptosis of CD8+ T cells is sufficient for their phagocytosis, we pretreated CD8+ T cells with dexamethasone to induce apoptosis before coculture with M-DC/IL-10 in the presence of INF-γ. The degree of apoptosis of CD8+ T cells correlated with their phagocytosis (Fig. 4B). However, CD8+ T cells pretreated with dexamethasone, which would induce apoptosis and possibly attenuate INF-γ production, were rendered less efficiently phagocytosed by M-DC/IL-10 compared with nontreated CD8+ T cells. In contrast, anti-CD3 pretreated CD8+ T cells were more efficiently phagocytosed by M-DC/IL-10 than control CD8+ T cells (Fig. 7A), indicating the importance of pre-activation of CD8+ T cells for them to be phagocytosed. These two lines of evidence suggest that timely apoptosis of previously activated CD8+ T cells on encounter with M-DC/IL-10 is necessary, but CD8+ T cells already predestinated for apoptosis may not be critical for their phagocytosis by M-DC/IL-10. High-level expression of PD-1 is detected on chronically activated CD8+ T cells (42). Thus, our view may explain a potential mechanism for PD-1–mediated exhaustion of previously activated CD8+ T cells.

It was not easy to estimate exact numbers of accumulated CD8+ T cells taken up by M-DC/IL-10 during a 5-d coculture, mainly because the phagocytosed CD8+ T cells did not remain intact enough to be counted. Because M-DC/IL-10 gained MFI as a result of phagocytosis of green fluorescent CFSE+ CD8+ T cells, we attempted to estimate numbers of CD8+ T cells taken up by M-DC/IL-10 in the presence of INF-γ using cyometric analysis for MFI. Based on the assumption that CD8+ T cell volume was ∼1/27 of M-DC based from our microscopic observation of the diameter of a CD8+ T cell being ∼1/3 that of M-DC, we estimated the numbers of CD8+ T cells phagocytosed by using methods described in Materials and Methods. Our results showed that ∼1.3 and 10.3 CD8+ T cells were phagocytosed by M-DC/IL-10/INF-γ, respectively, at CD8+ T cell/M-DC ratios of 2 and 20.

In summary, we demonstrate that PD-1 expression is induced on CD8+ T cells on coculture with M-DC/IL-10 or M-DC/IL-10/INF-γ cells that were developed from human CD14+ monocytes in vitro by a combination of IL-4, M-CSF, IL-10, and INF-γ. We also found that M-DC/IL-10/INF-γ, which express high levels of PD-L1, act as phagocytic cells for CD8+ T cells and, to a lesser extent, CD4+ T cells. Inhibition of phagocytosis by neutralizing anti–PD-L1 indicates that PD-1/PD-L1 interaction mediates this phagocytic activity. This study suggests a role for M-DC/IL-10 cells in downmodulating CD8+ T cell function in response to INF-γ by phagocytosis, as modeled in Fig. 8.

Discussion

It is well accepted that DCs can induce tolerance, as well as immuno- immuity, but underlying mechanisms for induction or tolerance of immunity by DCs remain unclear. Immature, or alternatively differentiated, DCs are probably primary cells for tolerance responses. T cell exhaustion is defined as gradual loss of effector functions, finally resulting in deletion of virus-specific T cells (39, 42). Loss of functional CD8+ T cell response to persistent viral
infection and tumors results from active inhibitory DCs (47–49). One type of inhibitory DC for inducing T cell tolerance is a MDSC (22, 50) and another is an immature MΦ (21). PD-L1 on MDSC has been implicated in induction of exhaustion of PD-1–expressing virus-specific T cells (9, 21). An important question yet to be answered was what factors modulate respective induction of PD-L1 and PD-1 on MDC and CD8+ T cells.

Our study provides evidence that orchestrated serial stimulation processes are required for induction of PD-L1 and PD-1, respectively, on tolerogenic DCs and CD8+ T cells. M-DCs are characterized by a skewed balance toward high IL-10 and low IL-12 secretion (40). The anti-inflammatory environment facilitated by IL-10 may be critical for inducing PD-L1 on the cell surface and an alternative differentiation process toward tolerance-inducing DCs (Fig. 2). On interaction with the PD-L1–expressing DCs, CD8+ T cells enhanced PD-1 expression levels as demonstrated in Fig. 1A. IFN-γ secreted by encountering CD8+ T cells may further increase PD-L1 levels on DCs primed with M-CSF and IL-10 (Fig. 2). The high level of PD-L1 expressed on the DCs may allow tight interactions between DCs and CD8+ T cells, and induce growth arrest or cell death of CD8+ T cells. The PD-1/PD-L1 signaling pathway may then trigger IFN-γ-producing CD8+ T cells to initiate apoptosis and consequently phagocytic response. Phagocytosis was significantly decreased by neutralizing anti–PD-L1. This indicates that elevated PD-L1 expression on M-DC/IL-10 by IFN-γ plays an important role in eliciting phagocytic activity (Figs. 5, 6). Thus, the local cytokine micromilieu, enriched with M-CSF and IL-10, may control functional differentiation of DCs from immunostimulatory to tolerogenic cells targeting IFN-γ–producing CD8+ T cells for phagocytosis. Although PD-1 acts on both CD4+ and CD8+ T cell populations as a coinhibitory receptor (51), CD8+ T cells were more susceptible, compared with CD4+ T cells, in response to phagocytosis (Fig. 5C, 6D). This may be because CD8+ T cells express PD-1 more readily or respond to PD-L1 more sensitively compared with CD4+ T cells.

Our results emphasize the importance of IFN-γ, a proinflammatory cytokine, for phagocytic clearance of CD8+ T cells as an additional level of CD8+ T cell tolerance. IFN-γ is a proinflammatory cytokine produced mainly by type 1 effector T cells and is required for CD8+ T cell cytotoxic functions. Thus, it seems paradoxical that phagocytic deletion of CD8+ T cells depends on IFN-γ. We believe that IFN-γ is important not only for type 1 effector T cell immune responses, but also for clearing effector CD8+ T cells by MDSC. Therefore, the phagocytic activity we observed in this study may take place in vivo to keep IFN-γ–producing CD8+ T cells in check to prevent excessive or continuous immune responses. TNF-α failed to develop phagocytic activity in M-DC/IL-10 cells, but instead induced cell death (data not shown). In mouse models, IFN-γ has been reported to negatively regulate CD8+ T cell response by promoting cell death mediated by PD-1, and blockade of IFN-γ augments the severity of graft-versus-host disease in allogeneic hematopoietic cell transplantation (52, 53). Furthermore, other mouse systems show that IFN-γ plays immunosuppressive roles in conjunction with MDSCs (24, 54).

Our study does not determine whether M-DC/IL-10 are relevant to certain DC subtypes in mice that show suppressive effects on CD8+ T cell. In a mouse model, treatment of animals with neutralizing anti–PD-L1 did not eliminate or reduce Gr-1+CD11b+ MDSC-induced CD8+ T cell tolerance (28), suggesting that PD-
phagocytic DCs against CD8+ T cells. IL-10 changes characteristics of M-DC/IL-10-derived M-DCs (M-DC/IL-10) with induction of elevated PD-L1 expression on M-DC/IL-10. Pretreatment of CD8+ T cells with dexamethasone or anti-CD3 affects responsiveness to phagocytosis by M-DC/IL-10. A, CD8+ T cells were incubated in IL-15 (20 ng/ml) with or without dexamethasone (10^{-7} M) or anti-CD3 coated to culture plates for 5 d. After CFSE staining, the CD8+ T cells were cocultured with M-DC/IL-10 in the presence of IFN-γ for 4 d. Cells were stained with anti-CD8 and anti-CD11c, and CD11c+ cells were analyzed for levels of CFSE by flow cytometry. Numbers in second quadrants represent percentage CFSE+ CD11c+ cells. This result is representative of four independent experiments. B, CD8+ T cells were incubated in IL-15 (20 ng/ml) for 5 d and subsequently stained with CFSE. CFSE+CD8+ T cells were cultured in the presence of IFN-γ for an additional 4 d without or with M-DC/IL-10 at ratios of T cells to M-DC/IL-10 of 2 and 20. M-DC/IL-10 and CD8+ T cells cultured separately or cocultured for 4 d were analyzed for MFI of CFSE by flow cytometry. Numbers of CD8+ T cells phagocytosed by M-DC/IL-10 were estimated from MFIs using the methods described in Materials and Methods. This result is representative of eight experiments.

L1 is not required for MDSC-mediated CD8+ T cell immune suppression. Therefore, the MDSCs identified as Gr-1+CD11b+ cells in the mouse may not be relevant to the M-DC we generated from human CD14+ monocytes in vitro. In another mouse study (29), it was reported that IFN-γ-stimulated monocyte-derived cells could suppress T cell immune responses by killing activated CD4+ T cells. However, this suppressive activity was accomplished by a caspase-dependent, but PD-1/PD-L1-independent, mechanism. However, phenotypes of the mouse IFN-γ-stimulated monocyte-derived cells were similar to those of our M-DC/IL-10/IFN-γ cells with high expression of PD-L1 and CD14. Thus, it would be of interest in the future to explore the possibility that M-DC/IL-10/IFN-γ cells may use caspase to promote apoptosis and/or phagocytosis of CD8+ T cells as seen in our study. The mouse IFN-γ-stimulated monocyte-derived cells were also shown to convert cocultured T cell populations to CD4+ CD25+Foxp3+ regulatory cells (29). We have previously demonstrated that M-DC were able to suppress CD4+ T cell proliferation (40), but it remains unclear whether M-DC have a capacity to generate CD4+FOXP3+ Tregs.

Prior IL-10 stimulation was critical for IFN-γ to be effective for developing phagocytic activity in M-DC/IL-10. In fact, IL-10 and IFN-γ are considered to play opposite roles, as anti-inflammatory and proinflammatory cytokines, but they may cooperate to elicit phagocytic activity against CD8+ T cells via M-DC, as long as IL-10 precedes IFN-γ stimulation. An important role of IL-10 in these processes is to block maturation of DCs (25), as shown in Fig. 3A. Importantly, immaturity of M-DC/IL-10 resulting from exposure to IL-10 may be an important conditioning event for subsequent IFN-γ to elicit phagocytic activity in M-DC/IL-10, indicating that strict sequential pathways starting from anti-inflammatory to inflammatory cytokine stimulation may be crucial for development of phagocytic DCs. The IL-10 effect depended on M-CSF for developing phagocytic activity. GM-DCs after IL-10 and IFN-γ stimulation showed little phagocytic activity even though they expressed relatively high levels of PD-L1.

Thus, M-CSF and IL-10 are required for guiding the direction of DC maturation from peripheral CD14+ monocytes to phagocytic DCs, rather than to immunostimulatory DCs. M-DC/IL-10 cells may be locked in as tolerogenic DCs at this stage and may be unable to convert to immunogenic DCs even in the presence of IFN-γ. This idea is supported by high expression of CD200R on MΦs, which delivers negative regulatory signals to induce tolerogenic MΦs and DCs (55), on M-DC/IL-10 and M-DC/IL-10/IFN-γ. Because IL-10 inhibits production of IFN-γ by CD8+ T cells, IL-10 and IFN-γ may rarely coexist at the same local sites. Thus, M-DC/IL-10 and IFN-γ-producing CD8+ T cells may be derived from supposedly distinct sites, enriched, respectively, with anti-inflammatory IL-10 or proinflammatory IL-12. Phagocytosis may arise in vivo when M-DC/IL-10 are recruited to proinflammatory sites where IFN-γ-producing CD8+ T cells prevail. Alternatively, this may happen as well when IFN-γ-producing CD8+ T cells migrate to the immune evasive sites such as tumor microenvironments enriched with M-DC/IL-10. In any event, PD-1 and PD-L1, respectively expressed on target and phagocytic cell surfaces, may be important cognate receptors for phagocytosis.

M-DC/IL-10 and M-DC/IL-10/IFN-γ cells show high expression levels of CD14 and CD200R, as well as CD11c, a marker for myeloid DCs. Although CD14 is expressed largely on MΦs and CD200R is expressed on both activated MΦs and DCs (55). M-DC/IL-10 and M-DC/IL-10/IFN-γ cells were less adherent cells, compared with prototype MΦs generated by M-CSF in the absence of IL-4, which favors DC differentiation. Therefore, although we initially used the term DCs for M-DCs generated in vitro from CD14+ monocytes by M-CSF and IL-4, which shares many typical DC surface markers on LPS stimulation (40). M-DC/IL-10 and M-DC/IL-10/IFN-γ cells may represent unique MΦ-like DCs or possibly an alternatively activated MΦ or an anti-inflammatory M2 MΦ subset (55). M-DC/IL-10 cells may be relevant to decidual MΦs, which are involved in maternal immunosuppression against fetus, because decidual MΦs produce high levels of IL-10 with cell surface expression of PD-L1 and CD200R, but low levels of costimulatory molecule CD86 (56–59).

**FIGURE 7.** Pretreatment of CD8+ T cells with dexamethasone or anti-CD3 affects responsiveness to phagocytosis by M-DC/IL-10. A, CD8+ T cells were incubated in IL-15 (20 ng/ml) with or without dexamethasone (10^{-7} M) or anti-CD3 coated to culture plates for 5 d. After CFSE staining, the CD8+ T cells were cocultured with M-DC/IL-10 in the presence of IFN-γ for 4 d. Cells were stained with anti-CD8 and anti-CD11c, and CD11c+ cells were analyzed for levels of CFSE by flow cytometry. Numbers in second quadrants represent percentage CFSE+ CD11c+ cells. This result is representative of four independent experiments. B, CD8+ T cells were incubated in IL-15 (20 ng/ml) for 5 d and subsequently stained with CFSE. CFSE+CD8+ T cells were cultured in the presence of IFN-γ for an additional 4 d without or with M-DC/IL-10 at ratios of T cells to M-DC/IL-10 of 2 and 20. M-DC/IL-10 and CD8+ T cells cultured separately or cocultured for 4 d were analyzed for MFI of CFSE by flow cytometry. Numbers of CD8+ T cells phagocytosed by M-DC/IL-10 were estimated from MFIs using the methods described in Materials and Methods. This result is representative of eight experiments.

**FIGURE 8.** A model for phagocytic response of exhausted CD8+ T cells to M-DC/IL-10/IFN-γ. M-CSF is a key cytokine for the development of phagocytic DCs against CD8+ T cells. IL-10 changes characteristics of DCs with an enhanced responsiveness to IFN-γ for an ability to induce PD-1 on CD8+ T cells. Subsequent IFN-γ stimulation further modifies IL-10-derived M-DCs (M-DC/IL-10) with induction of elevated PD-L1 expression on M-DC/IL-10/IFN-γ. CD8+ T cells able to produce IFN-γ may further stabilize expression of PD-L1 on M-DC/IL-10/IFN-γ. Tight interactions between CD8+ T cells and M-DC/IL-10/IFN-γ cells through PD-1 and PD-L1 may induce apoptosis of CD8+ T cells, which are eventually phagocytosed by M-DC/IL-10/IFN-γ.
Our confocal microscopic images indicate that many CD8+ T cells remain associated with M-DC/IL-10 even after washing the glass slides with PBS, especially in the presence of IFN-γ during the coculture (Fig. 5C). Moreover, IFN-γ increased many fluorescent speckles in M-DC/IL-10 cells cocultured with CFSE-stained CD8+ T cells, presumably derived from CFSE-stained CD8+ T cells. We noticed many vacuoles created on the surface of M-DC/IL-10 only when both CD8+ T cells and IFN-γ were present (Fig. 5B). Many of these vacuoles appeared to be occupied by CD8+ T cells. This suggests that CD8+ T cells may be directly involved in creating the vacuoles. Exhausted CD8+ T cells may continue to produce IFN-γ (3), and the final fate of the IFN-γ-producing CD8+ T cells expanded by chronic virus infection is unclear. The symptomatic CD8+ T cell exhaustion by HIV, as well as other infections, is mainly attributed to PD-1 and IL-10 (32).

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Disclosures
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References


