

CD8 α^+ and CD8 α^- Subclasses of Dendritic Cells Direct the Development of Distinct T Helper Cells In Vivo

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Summary

Cells of the dendritic family display some unique properties that confer to them the capacity to sensitize naive T cells in vitro and in vivo. In the mouse, two subclasses of dendritic cells (DCs) have been described that differ by their CD8 α expression and their localization in lymphoid organs. The physiologic function of both cell populations remains obscure. Studies conducted in vitro have suggested that CD8 α^+ DCs could play a role in the regulation of immune responses, whereas conventional CD8 α^- DCs would be more stimulatory. We report here that both subclasses of DCs efficiently prime antigen-specific T cells in vivo, and direct the development of distinct T helper (Th) populations. Antigen-pulsed CD8 α^+ and CD8 α^- DCs are separated after overnight culture in recombinant granulocyte/macrophage colony-stimulating factor and injected into the footpads of syngeneic mice. Administration of CD8 α^- DCs induces a Th2-type response, whereas injection of CD8 α^+ DCs leads to Th1 differentiation. We further show that interleukin 12 plays a critical role in Th1 development by CD8 α^+ DCs. These findings suggest that the nature of the DC that presents the antigen to naive T cells may dictate the class selection of the adaptative immune response.

Key words: primary response • T helper cell type 1/type 2 balance • interleukin 12 • tolerance • memory

Since their discovery 25 years ago, dendritic cells (DCs)¹ have gained increasing interest from immunologists, as they are specialized in the capture, processing, and transport of the antigen to lymphoid organs where they probably sensitize antigen-specific naive T lymphocytes (1). More recently, Shortman and colleagues developed a procedure that incorporated a step to dissociate DC-lymphocyte complexes, leading to the discovery of a new subset of DCs that expresses a CD8 $\alpha\alpha$ homodimer and limits the proliferation of CD4⁺ T cells in vitro by Fas-mediated death (2, 3). Based on these observations and on a recent report that T cell area DCs express high levels of self-peptides (4), it was suggested that the CD8 α^+ cells could play a role in peripheral tolerance in vivo, whereas conventional CD8 α^- DCs would initiate immune responses. This hypothesis was challenged by recent reports that IL-12 was produced by CD8 α^+ rather than CD8 α^- DCs (see below). This prompted us to assess the function of both subclasses

in vivo by injecting purified DCs, pulsed extracorporeally with antigen, into the footpads of syngeneic mice and analyzing the immune response of LN cells.

Materials and Methods

Mice. Balb/c mice were purchased from Iffa-Credo. Balb/c IL-12 p40^{-/-} mice were provided by Dr. J. Magram (Hoffmann-La Roche, Nutley, NJ [5]). All mice were maintained in our pathogen-free facility and used at 7–9 wk of age.

Purification and Stimulation of DCs. DCs were purified as shown previously (6), except that spleen cells were digested with collagenase, further dissociated in Ca²⁺-free media in the presence of EDTA, separated into low- and high-density fractions on a Nycodenz gradient, and cultured overnight with 15 ng/ml rmGM-CSF, as described (3). DCs were pulsed with antigen (30 μ g/ml KLH) during overnight culture (6). The CD8⁻ or CD8⁺ status was maintained after culture in GM-CSF-containing medium for 1–5 d (7; and our unpublished observations), suggesting that CD8 α is a stable lineage marker. After overnight culture, nonadherent cells (containing at least 90% DCs, as assessed by morphology and specific staining, using anti-CD11c mAb, N418; reference 8) were incubated with anti-CD8 α -coupled microbeads

¹Abbreviation used in this paper: DC, dendritic cell.

and separated according to CD8 α expression by two passages over a MACS[®] column (Miltenyi Biotec). The CD8 α ⁻ DCs were further enriched by incubation with anti-CD11c-coupled microbeads and positive selection over a MACS[®] column. Alternatively, nonadherent cells collected after overnight culture were separated according to CD8 expression by FACS[®] sorting; in brief, cells were double stained for CD11c expression using FITC-conjugated N418 and for CD8 α expression using biotin-conjugated anti-CD8 α mAb (PharMingen) followed by PE-streptavidin. The cells were gated based on characteristic forward and side light scatter, and two populations (CD11c⁺CD8 α ⁺ and CD11c⁺CD8 α ⁻) were sorted on a FACS Vantage[®] (Becton Dickinson). The proportion of CD8 α ⁺ to CD8 α ⁻ DCs at the end of the purification steps was 10–15% in all experiments performed.

Induction of IL-12 from DC Subsets. Low-density spleen cells (see above) were enriched for CD11c expression and further separated according to CD8 α expression using a Multisort anti-FITC kit (Miltenyi Biotec). Cells were cultured overnight with pansorbin (20 μ g/ml; Calbiochem) plus IFN- γ and GM-CSF (20 ng/ml each), and the supernatant was assayed for IL-12 p70 using ELISA from Genzyme. The detection limit was 8 pg/ml.

Immunization Protocol. Antigen-pulsed DCs were washed in RPMI 1640 and administered at a dose of 3×10^5 cells into the hind footpads, according to a protocol described by Inaba et al. (9). When indicated, some groups of animals were treated with 1 mg anti-CD4 mAbs (GK 1.5), to selectively deplete CD4⁺ T cell subset in vivo, as described previously (10). Some mice were injected daily with 0.2 μ g i.p. rIL-12 on days 0, 1, 2, and 3. Draining popliteal LNs were harvested 5 d after DC injection.

In Vitro Assays. LN cells were cultured in Click's medium supplemented with 0.5% heat-inactivated mouse serum and additives. The proliferation was measured as thymidine incorporation during the last 16 h of a 4-d culture. Culture supernatants were assayed for IL-2 after 24–48 h, and for IFN- γ , IL-4, IL-5, and IL-10 after 96 h of incubation. IL-2, IFN- γ , and IL-10 were measured as described (11). IL-4 and IL-5 were quantitated by two-site ELISA from Genzyme and PharMingen, respectively. The detection limits were 0.05 U/ml for IL-2, 1 ng/ml for IFN- γ , 15 pg/ml for IL-4, 2 U/ml for IL-5, and 0.3 ng/ml for IL-10.

Results

CD8⁺ and CD8⁻ DCs, Pulsed In Vitro with Antigen, Prime T Cells In Vivo. DCs were purified from spleens, pulsed with KLH during overnight culture (6), and further separated according to CD8 α expression by FACS[®] sorting or by positive/negative selection on MACS[®]. Reanalysis of the sorted cell populations confirmed purity >99% (FACS[®]) or 97% (MACS[®]). 3×10^5 DCs were injected into the hind footpads of syngeneic mice, and the popliteal LNs were harvested 5 d later. The data in Fig. 1 a indicate that administration of purified CD8 α ⁺ or CD8 α ⁻ DCs, or both, loaded ex vivo with antigen, resulted in T cell priming, as assessed by KLH-dependent proliferation in culture. The proliferative response of LN cells from mice injected with CD8 α ⁺ DCs was consistently higher compared with animals primed with CD8 α ⁻ DCs. T cell priming was prevented by treatment of mice with neutralizing anti-CD4 mAbs, showing that the KLH-specific response was dependent on CD4⁺ T lymphocytes.

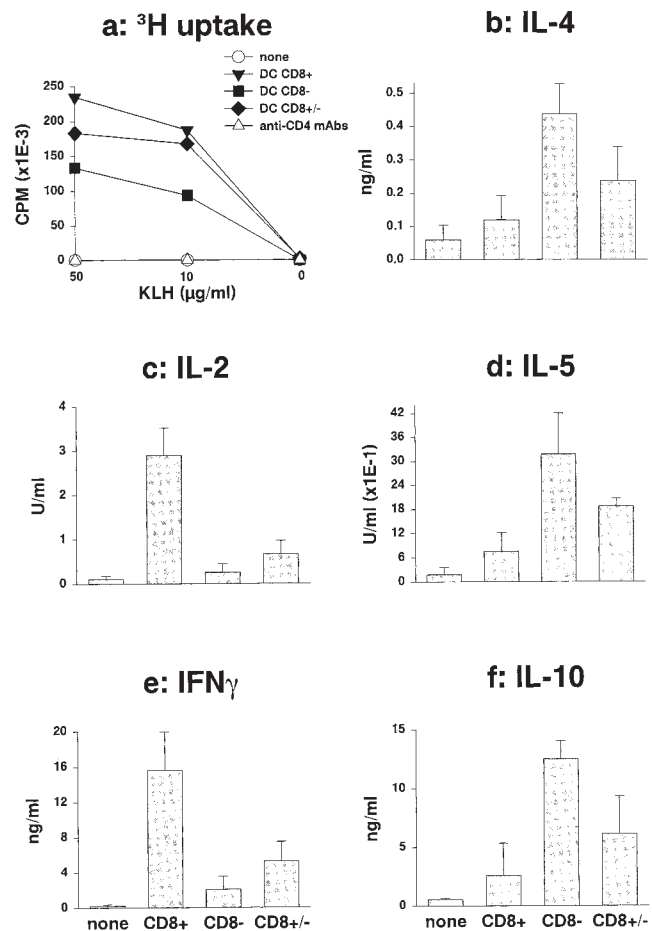


Figure 1. CD8 α ⁺ and CD8 α ⁻ DCs prime distinct Th cells in vivo. LN cells from mice injected with either subset or a combination (at a proportion of 1 CD8 α ⁺ to 10 CD8 α ⁻) 5 d earlier were cultured without antigen or with 50 (a) or 10 (a–f) μ g/ml KLH. Some mice were injected with 1 mg anti-CD4 mAbs before priming. Proliferation and lymphokine production were measured as indicated in Materials and Methods. Figure summarizes the results (mean \pm SD) obtained in five independent experiments.

The Subclasses of DCs Induce the Development of Distinct Th Populations. We next analyzed the cytokines (12) released by LN cells from mice primed with either subset in vivo. The data in Fig. 1 indicate that the subclasses of DCs have the potential to differentially skew cytokine production towards Th1 and Th2 tendencies (12): CD8 α ⁻ DCs induced the activation of cells secreting high levels of IL-4, IL-5, and IL-10 and low levels of IL-2 and IFN- γ , whereas CD8 α ⁺ DCs sensitized cells producing IL-2 and IFN- γ , but little IL-4, IL-5, and IL-10. Unseparated splenic DCs (11; and data not shown) or a combination of both subsets (at a proportion of 1 CD8 α ⁺ to 10 CD8 α ⁻) induced the activation of helper cells secreting a large array of lymphokines.

Analysis of the Memory Response. These results indicate that both CD8 α ⁺ and CD8 α ⁻ subsets of DCs can act as adjuvant of the immune response and differentially regulate the development of CD4⁺ Th cells. As in vitro data suggested that CD8 α ⁺ DCs could kill Fas-expressing cells (3),

T lymphocytes may undergo Fas-mediated apoptosis once they are activated, i.e., later during the primary response. Therefore, we tested whether an anamnestic response was developed. Unseparated, KLH-pulsed DCs were injected into the hind footpads of mice that were untreated or immunized 14 d earlier with various DC populations. LN cells were harvested 2 d later and cultured with or without antigen. The data in Fig. 2 a indicate that a memory response was induced in all preimmunized groups, as assessed by antigen-specific T cell proliferation of LN cells from mice that received two injections of DCs, but not in groups that received only one injection. Of note, the cytokine profiles were determined by the subclass of DCs used to prime animals: mice injected with CD8 α^+ or CD8 α^- DCs and boosted with unseparated DCs displayed a secondary response of Th1 and Th2 type, respectively (Fig. 2). These findings indicate that both DC subsets induce the development of memory helper cells that upon recall with the same antigen differentiate into distinct helper populations.

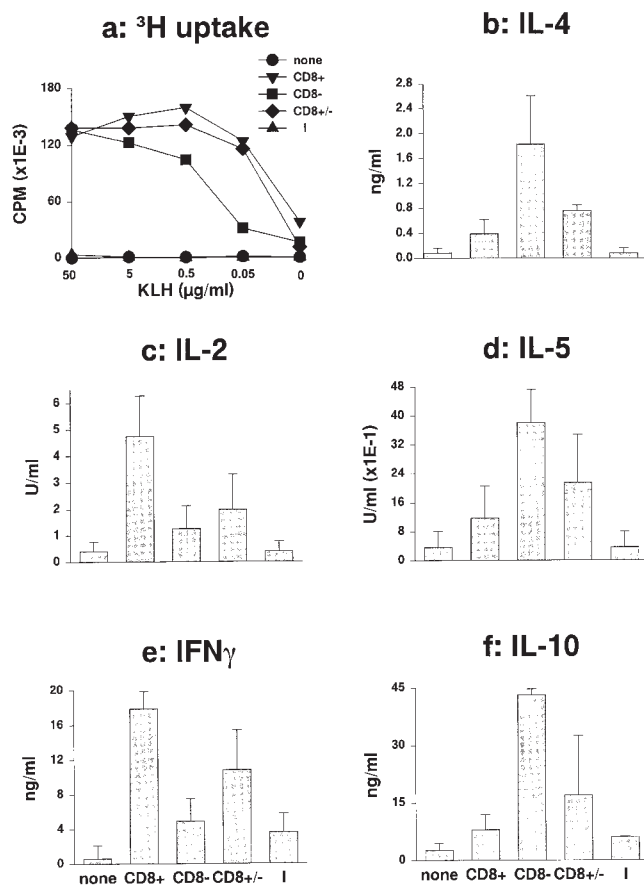


Figure 2. CD8 α^+ and CD8 α^- DCs induce distinct memory responses in vivo. Balb/c mice were either left untreated (I) or were primed with KLH-pulsed CD8 α^+ , CD8 α^- , or the combination 14 d earlier, and boosted with unseparated DCs, pulsed with the same antigen. LN cells were harvested 2 d later and cultured with (a) graded doses or (b–f) 10 $\mu\text{g/ml}$ KLH. Proliferation (a) and lymphokine production (b–f) were measured as indicated in Materials and Methods. Figure summarizes the results (mean \pm SD) obtained in four independent experiments.

Role of IL-12. There is evidence that the maturation of Th precursors into biased Th1 or Th2 populations is strongly influenced by cytokines in the environment (13). In particular, IL-12 appears as the dominant cytokine driving the differentiation of Th1 lymphocytes in vitro and in vivo (14, 15). We found that CD8 α^+ DCs produced high levels of IL-12 heterodimer upon stimulation, whereas CD8 α^- DCs secreted little if any IL-12 (Table I). The role of IL-12 in Th1 priming was further documented by the observation that CD8 α^+ DCs isolated from mice deficient for IL-12 (5) induced little IFN- γ and intermediate levels of IL-4 when injected into syngeneic mice, compared with CD8 α^+ DCs from wild-type mice (Fig. 3, a and b). Conversely, coinjection of rIL-12 and antigen-pulsed CD8 α^- DCs resulted in the development of a polarized Th1-type immune response (Fig. 3, c and d).

Discussion

There is increasing evidence that protection against parasites or infectious diseases relies on the character of the immune response, i.e., the Th1/Th2 balance. In particular, Th1 cells are important effectors involved in the eradication of intracellular infectious pathogens, whereas Th2 lymphocytes are efficient to eliminate extracellular parasites. Importantly, the development of the inappropriate Th subset not only fails to eradicate the pathogen but can cause immunopathology (for a review, see reference 13). Therefore, it is of major interest to identify the factors that influence the differentiation of distinct Th cell subsets in vivo. We show herein that two subclasses of DCs differentially regulate the development of Th cells secreting discrete sets of lymphokines: CD8 α^+ DCs direct the differentiation of Th1 cells, whereas CD8 α^- DCs induce Th2-type responses in vivo. We further show that both DC subclasses induce memory responses, and that the activation of Th1 responses by CD8 α^+ DCs correlates with their production of IL-12 p70 heterodimer.

Table I. Production of IL-12 by DCs

	IL-12 p70 (pg/ml)			
	Untreated		Pansorbin + IFN- γ + GM-CSF	
	CD8 α^-	CD8 α^+	CD8 α^-	CD8 α^+
Exp 1	11.5	<8.0	13.8	190.1
Exp 2	12.7	11.1	33.3	812.2
Exp 3	17.7	19.8	93.3	854.8

Low-density spleen cells were sorted according to CD8 α expression and cultured with or without pansorbin plus IFN- γ plus GM-CSF. Supernatants were assayed for IL-12 p70 production 24 h later. Exp, experiment.

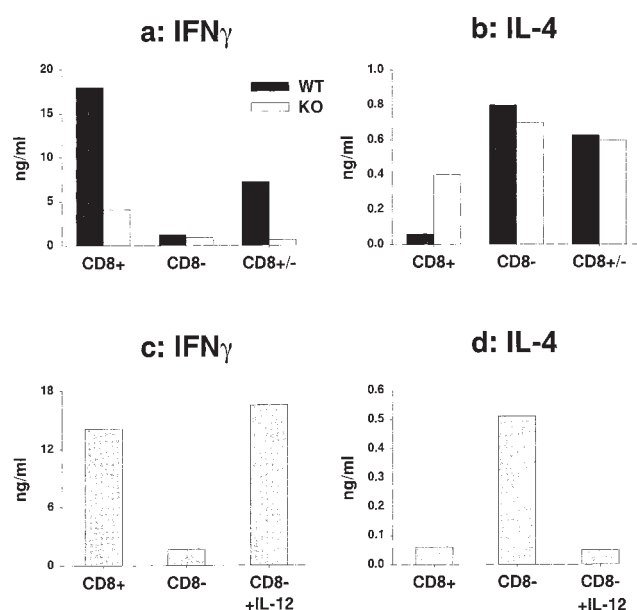


Figure 3. The priming of Th1 cells by CD8 α^{+} DCs is IL-12 dependent. (a and b) CD8 α^{+} or CD8 α^{-} DCs or both subsets, isolated from p40-deficient (KO) or wild-type (WT) mice, were loaded with KLH and injected into the footpads of Balb/c mice. (c and d) Balb/c mice were injected in the footpads with CD8 α^{+} or CD8 α^{-} DCs with or without four daily injections of 200 ng i.p. rmlIL-12. LN cells were harvested 5 d later and cultured with 10 μ g/ml antigen. The production of IFN- γ and IL-4 was measured in the supernatants, as indicated in Materials and Methods. Similar data were obtained in all four experiments performed.

Our data are in agreement with recent reports that CD8 α^{+} DCs are the source of IL-12. In Flt3 ligand-treated mice, a DC subset containing a majority of CD8 α^{+} cells was shown to secrete very high levels of IL-12 (16). Similarly, most DCs exposed to soluble *Toxoplasma gondii* tachyzoite extract in vivo that stained for IL-12 p40 were shown to belong to the CD8 α subset (17). In addition, our results suggest that the same cells, i.e., the transferred DCs, function as APCs and IL-12-producing cells, as DCs from p40 knockout mice have lost the capacity to induce a Th1-type response in a wild-type host. These observations and other studies suggest that IL-12 is a dominant factor in directing the development of Th1 cells producing high levels of IFN- γ in vitro and in vivo, although other factors may affect Th subset development (13). Of note, several reports suggest that IL-12 production by DCs requires their prior activation and that the amount of IL-12 depends on the mode of activation. Reis e Sousa and collaborators reported a CD40L- and IFN- γ -independent production of IL-12 after infection with *T. gondii* or injection of LPS (17). Koch et al. (18) found that ligation of CD40 and MHC class II molecules independently triggered IL-12 production by murine DCs. Similarly, CD40L was found to be the most effective stimulus to induce IL-12 by DCs generated from human peripheral blood monocytes (19). Therefore, it is tempting to speculate that, in our model, the transferred antigen-pulsed DCs are triggered to release IL-12 upon antigen-specific interaction with T lymphocytes in vivo. Ex-

periments are underway to define the role of CD40-CD154 interaction in Th1 priming and to compare the IL-12 produced by DCs at various stages of maturation.

There is some evidence that CD8 α^{+} and CD8 α^{-} DCs belong to distinct lineages. Shortman and colleagues have found in intravenous transfer studies that CD8 α serves as a marker of the DC progeny of the low CD4 precursor, in both the thymus and the spleen of irradiated recipients, thereby suggesting that CD8 α^{+} DCs are of lymphoid origin (20, 21). Conversely, the CD8 α^{-} DCs would be of myeloid origin, as they are relatives of monocytes and macrophages and are GM-CSF dependent (21, 7). Both classes of DCs maintain their CD8 α^{-} or CD8 α^{+} status in culture in the presence of GM-CSF, and therefore appear as stable distinct lineages (7). Of note, CD8 α remained a stable marker on DCs cultured for 5 d in the presence of GM-CSF, IFN- γ , and/or activated T cells (our unpublished observations).

A recent study by Pulendran et al. (21a) confirmed that both subsets of DC differentially regulated the development of T helper cells in vivo. They showed that the lymphoid-related subsets induced high levels of IFN- γ and IL-2, but little Th2 cytokines, whereas the myeloid-related subset induced large amounts of IL-4 and IL-10, in addition to IFN- γ and IL-2. The CD8 α^{+} population used in the present study is CD11c $^{+}$ CD11b dull or $^{-}$, and therefore is likely to represent the lymphoid-related population D/E described by Pulendran and colleagues (16). By contrast, the CD8 α^{-} DCs are CD11c $^{+}$ CD11b $^{+}$ and resemble the myeloid-related DC subset referred to as population C (data not shown). The distinct regulation of the IFN- γ and IL-2 synthesis, compared with our study, could be related to differences in the purification procedures, in the maturation state of the DCs transferred (fresh versus cultured DCs), in the form of antigen (peptide versus protein), and/or in the precursor frequency of antigen-reactive T cells (TCR transgenic versus wild-type mice). It is noteworthy that injection of CD8 α^{+} and CD8 α^{-} DCs isolated from mice treated with Flt3 ligand (provided by Dr. C. Maliszewski, Immunex, Seattle, WA) induced the development of similar Th cells compared with DCs purified from untreated mice, suggesting that administration of Flt3 ligand did not alter DC function (data not shown).

Two reports have shown that the cell population containing the highest proportion of CD8 α^{+} cells was consistently less efficient at stimulating the proliferation of antigen-specific cells in vitro (3, 22). A ligand for Fas was demonstrated on the surface of CD8 α^{+} but not CD8 α^{-} DCs, and the suboptimal activation of T cells by CD8 α^{+} DCs was associated with marked T cell apoptosis via Fas engagement (3). We show here that injection of pulsed CD8 α^{+} and CD8 α^{-} DCs induced equally strong T cell proliferative responses upon in vitro restimulation. Although we did not measure the expansion of T cells in situ, a difference between the in vitro and in vivo function of CD8 α^{+} DCs could be due to the segregation of cell populations into distinct geographic compartments in vivo after T cell activation (23) compared with the confined mi-

croenvironment in culture plates. Alternatively, it is possible that Th1-type responses, which could be deleterious, are controlled by a feedback mechanism involving Fas-mediated killing of T lymphocytes once activated (24, 25). Interestingly, there is evidence that CD95L may be a mediator of costimulation and inflammation as well as a death agonist (26–30): CD95L has been shown to recruit neutrophils and activate their cytotoxic machinery, leading to local inflammation (29). As inflammatory products seem to induce the maturation of DCs and their migration to lymphoid tissues (31, 32), inflammation may be crucial for the initiation of immunity. Experiments are underway to test whether FasL expression by CD8 α^+ is required for the induction of a Th1-type response in vivo.

It is intriguing that CD8 α^+ and CD8 α^- DCs seem to be located in distinct microenvironments of the spleen (16). In Balb/c mice, a majority of CD8 α^- DCs reside at the margin between the red and white pulp, whereas most CD8 α^+ DCs are present in the zones where T cells are located (our unpublished observations). Injection of LPS results in the redistribution (32) of both subsets into the T cell area (our unpublished observations), suggesting that both subsets

have migratory properties. It is notable that CD8 α^+ DCs express high levels of DEC-205, a multilectin-like receptor (33) which could be specific for carbohydrates that are common constituents of microbial cell walls. In addition, the expression of CD1d, an MHC-like molecule which presents antigens mainly derived from prokaryotes (34), has been shown to be highest on the CD8 α^+ DC subset (16). Therefore, it is tempting to speculate that the CD8 α^+ subset of DCs preferentially capture and present microbial antigens and elicit a Th1-type response.

In conclusion, two subsets of DCs, which differ by phenotype, functional, and histologic parameters, exist in the mouse, each one initiating a different class of response in vivo and thereby stimulating different effector mechanisms. Our data show that CD8 α^+ DCs drive the development of Th1-type immune responses, whereas CD8 α^- DCs induce the differentiation of Th2-type responses. These observations predict that CD8 α^+ DCs might not be exploited to induce tolerance in vivo and confer immune privilege to grafts, but instead may be attractive for eliciting therapeutic antitumor immunity.

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