Published in final edited form as:
J Immunol. 2009 March 1; 182(5): 2777–2785. doi:10.4049/jimmunol.0803172.

Ly6C^{low} Monocytes Differentiate into Dendritic Cells and Cross-Tolerize T Cells through PDL-1[1]

YuFeng Peng[*], **Yvette Latchman**[†,‡], and **Keith B. Elkon**[2,*,†]

[*] Division of Rheumatology, University of Washington, Seattle, WA 98195

[†] Department of Immunology, University of Washington, Seattle, WA 98195

[‡] Puget Sound Blood Center, Seattle, WA 98104; and Division of Hematology, University of Washington, Seattle, WA 98195

Abstract

Monocyte-derived dendritic cells are active participants during the immune response against infection, but whether they play a role in maintaining self-tolerance under steady-state conditions is not known. Here we investigated the differentiation of monocytes, their ability to ingest apoptotic cells, and their potential functionality in vivo. We observed that Ly6C (Gr-1)^{low} mature monocytes up-regulate their MHC II level in the spleen, express high levels of PDL-1 (programmed death ligand 1), and are more efficient than Ly6C^{high} immature monocytes in the ingestion of apoptotic cells in vivo. Sorted circulating Ly6C^{low} monocytes were able to cross-present both apoptotic cell-associated OVA and soluble OVA protein. Monocytes containing apoptotic cells can further differentiate into CD11c^{+}CD8α^{-} MHC II^{+} splenic dendritic cells that maintained high expression of PDL-1. Since wild-type but not PDL-1-deficient peripheral blood monocytes containing apoptotic cell-associated OVA suppressed the response to OVA immunization, PDL-1 expression was required for monocyte-mediated T cell tolerance. These observations demonstrate that Ly6C^{low} mature monocytes can promote tolerance to self Ag contained in apoptotic cells through a PDL-1-dependent mechanism.

The potential self Ag burden in the immune system is massive. The apoptotic death of billions of cells per day by immune cells alone (reviewed in Ref. 1) are thought to actively contribute to the maintenance of self-tolerance. Self Ags that are contained within apoptotic cells are efficiently captured, processed, and transported by immature tissue dendritic cells (DCs).[3] In vitro and in vivo studies have shown that ingestion and processing of apoptotic cell-associated Ags (cross-presentation) lead to CD8 T cell activation and tolerization (reviewed in Ref. 2). The effect of apoptotic cell uptake on APC functions has mainly been studied in vitro. The ingestion of apoptotic cells by macrophages and DCs suppresses their function through a variety of mechanisms, including induction of suppressive cytokines, repression of IL-12, and inhibition of NF-κB (3–5). The in vivo tolerization effect of apoptotic cells is, in part, attributed to the low expression of B7-1 and B7-2 on immature DCs that have ingested apoptotic cells (6), but additional mechanisms may exist.

[1]This work was supported by National Institutes of Health Grant AR48796 (to K.B.E.). Y.F.P. was supported in part by a Career Development Grant from the Arthritis Foundation.

2Address correspondence and reprint requests to Dr. Keith B. Elkon, Division of Rheumatology, University of Washington, 1959 NE Pacific Street, Box 356428, Seattle, WA 98195. E-mail address: E-mail: elkon@u.washington.edu.

Disclosures

The authors have no financial conflicts of interest.

[3]Abbreviations used in this paper: DC, dendritic cell; Apo, apoptotic cell; PDL, programmed death ligand.

As an important organ that helps in the disposal of apoptotic cells, the spleen is populated with macrophages and DCs that serve as efficient phagocytes. Both macrophages and DCs are heterogeneous populations (7). The main DC subpopulations that reside in the spleen are $CD8\alpha^{+}CD11b^{-}$ and $CD8\alpha^{-}$ $CD11b^{+}$, which are reported to have notable differences in localization and function (8). $CD8\alpha^{+}$ DCs are located mainly in the T cell zone, whereas most $CD8\alpha^{-}$ DCs are located in and around the marginal zone (8). In response to an inflammatory stimulus, $CD8\alpha^{+}$ DCs are the main DC population that secretes IL-12 (9). Due to their different abilities to process Ag, it has been proposed that $CD8\alpha^{+}$ DCs are preferred APCs for $CD8\alpha^{+}$ T cells and that $CD8\alpha^{-}$ DCs favor CD4 T cell activation (8). Earlier in vivo and in vitro studies have also shown that $CD8\alpha^{+}$ are more efficient than $CD8\alpha^{-}$ DCs in phagocytosis of dying cells (10,11). Although the mechanisms that result in this superiority remain unclear, it has led to the conclusion that $CD8\alpha^{+}$ DCs are the main DCs population in the spleen that mediates cross-presentation and cross-tolerance.

Both splenic-derived precursors and bone marrow macrophage/DC precursors, as well as circulating monocytes, have been reported to contribute to the generation of splenic DCs (12–15). Two major monocyte subsets in the mouse can be distinguished based on their expression of Ly6C (Gr-1), CCR2, and CX3CR1. The classical $Ly6C^{high}CCR2^{high}CX3CR1^{low}$ monocyte ($CD14^{+}$ $CD16^{-}$ in humans) is generally called “inflammatory monocytes” due to their preferential migration to sites of inflammation. $Ly6C^{low}$ $CCR2^{low}CX3CR1^{high}$ monocytes ($CD14^{int}CD16^{+}$ in humans) are regarded as “stationary” because they appear to be the only monocyte population that extravasates into tissues under steady-state conditions (15). The function of $Ly6C^{low}$ cells under steady-state conditions remains uncertain.

Since $Ly6C^{low}$ monocytes could play a homeostatic role, we compared the ability of $Ly6C^{high}$ and $Ly6C^{low}$ monocytes to ingest apoptotic cells in vivo and followed their fate in the spleen. We analyzed their phenotypic properties as well as their function in relation to immune tolerance.

Materials and Methods

Animals

C57BL/6, CD45.1 congenic mice, and $CX3CR1^{GFP/GFP}$ (CX3CR1/GFP) mice were purchased from The Jackson Laboratory. OT-I CD8 TCR transgenic mice were kindly provided by Dr. Michael Bevan (University of Washington). PDL-1 (programmed death ligand 1)-deficient mice were previously described (16). Animals were housed in a specific pathogen-free facility and maintained according to approved protocol by the Institutional Animal Care and Use Committee at the University of Washington.

Abs and reagents

Abs specific for CD8 (53.67), MHC II (M5/114), CD11c (N418), CD11b (M1/70), Gr-1 (RB6 – 8C5), CD45.1 (A20), and CD45.2 (104) were purchased from BioLegend; anti-PDL-2 (TY25), PDL-1 blocking Ab (MIH-5), anti-33D1 were from eBioscience; Vβ5 (MR9-4) was from BD Biosciences; and anti-F4/80 (CI:A3-1) was from Serotec. 3D6, an Ab specific for marginal zone macrophage (CD169), was a kind gift from Dr. Simon Gordon (Oxford University, U.K.). The OVA peptide (257–264) was purchased from AnaSpec, and the purified OVA protein was from Calbiochem. OVA protein was depleted of LPS by the Triton X-100 procedure so that the final concentration was <0.6 EU as determined by the *Limulus* amebocyte lysate test (E-Toxate; Sigma-Aldrich).

Preparation of apoptotic cells

Thymocytes were exposed to 60 mJ/cm^2 UV light by a cross-linker and subsequently labeled with either PKH67 (Sigma-Aldrich) or CM-Dil (Invitrogen) for 1 h. Cells were washed extensively with PBS and injected into mice by the i.v. route. More than 80% of cells become annexin V-positive 4 h after exposure. To prepare apoptotic cells containing OVA protein, thymocytes were loaded with Ag by the osmotic shock method (17).

Transfer of peripheral blood cells and functional analysis

To investigate the function of apoptotic cell-containing monocytes, peripheral blood was collected 20 –24 h after apoptotic cell injection. RBCs were lysed and pooled samples from five mice were transferred to a new host. One week later, the recipient was challenged s.c. with 100 μg of OVA emulsified with CFA. Spleen cells were collected 1 wk later and restimulated with OVA peptide (257–264) for 6 h. Production of IFN-γ by CD8 T cells was assessed by intracellular staining and flow cytometry analysis (see below).

Immunofluorescence

Spleen samples were frozen in OCT. Tissue sections (8 μm) were fixed in ice-cold acetone and stained with biotinylated anti-CD11c, anti-CD4, and anti-TCR α-chain followed by streptavidin-Texas Red or streptavidin-Cy5 (Jackson ImmunoResearch Laboratories). Marginal zone macrophages were detected by the 3D6 mAb followed by rhodamine RedX-labeled Fab fragment of goat anti-rat IgG Ab (Jackson ImmunoResearch Laboratories). To visualize apoptotic cells in CX3CR1/GFP mice, spleen samples were fixed in 4% paraformaldyde with 10% sucrose for 6 h to preserve GFP before embedding.

Cross-presentation of apoptotic cell-associated OVA by Ly6C^{low} monocytes in vitro

Circulating CD11c$^+$CD11b$^+$CD8α^- PDL-1^{high} monocytes were sorted by flow cytometry and incubated with either 1 × 10^6 OVA-loaded apoptotic cells or soluble OVA (100 μg/ml) and CFSE labeled OT-I T cells. The dilution of CFSE in OT-I cells was analyzed at day 3.

Intracellular staining of cytokines

Ex vivo cytokine production by OT-I T cells was evaluated by incubating spleen cells with 1–2 μM OVA peptide in the presence of GolgiStop (BD Biosciences). Cells were fixed and permeabilized according to the manufacturer's instructions. The level of intracellular IFN-γ was detected by anti-IFN-γ allophycocyanin.

Results

Circulating Ly6C^{low}CD11c^{int} monocytes ingest apoptotic cells and express high levels of PDL-1

Efficient uptake of apoptotic cells is a well-known characteristic of macrophages and DCs. It is less clear how other types of phagocyte, such as monocytes, contribute to the disposal of apoptotic cells in vivo. Since blood is a relatively pure source of monocytes, we first asked whether circulating monocytes can take up apoptotic cells and, if so, what was their subsequent fate. Peripheral monocytes compose two major populations: Ly6C^{high}CX3CR1^{low} CCR2$^+$CD11c$^-$ and Ly6C^{low}CX3CR1^{high}CCR2$^-$CD11c^{int} (15). Using CX3CR1/GFP mice, where these two monocyte subsets can be distinguished by the intensity of GFP expression (15), we injected CM-Dil (red)-labeled syngenic apoptotic thymocytes i.v. and examined apoptotic cell uptake by circulating blood monocytes. As shown in Fig. 1*A*, at 20 h after injection, most of the apoptotic cells were found within the Ly6C^{low}GFP^{high}CD11c$^+$ population. The preferential ability of Ly6C^{low}CD11c$^+$ monocytes to ingest apoptotic cells was further confirmed by using F4/80 as a surface marker for monocytes (Fig. 1*B*).

To further characterize the phenotypic properties of circulating monocytes that ingested apoptotic cells, we examined their surface expression of the costimulatory/inhibitory markers B7-1, B7-2, PDL-1, and PDL-2 at 20 h postinjection. The apoptotic cell-containing monocytes in the circulation expressed high levels of PDL-1, whereas they were essentially negative for PDL-2, B7-1, B7-2, and ICOSL expression (Fig. 2*A*). The high expression of PDL-1 was associated with the $CD11c^{+}CX3CR1^{high}Ly6C^{low}$ mature monocyte phenotype (Fig. 2*B*). To directly compare the phagocytic abilities between immature and mature monocytes, we sorted blood monocytes based on their CD11c, CD11b, and PDL-1 expression and incubated purified populations with PKH67-labeled apoptotic cells in vitro. As shown in Fig. 2*C*, $PDL\text{-}1^{high}$ mature monocytes were more efficient than $PDL\text{-}1^{low}$ immature monocytes in the ingestion of apoptotic cells ($PDL\text{-}1^{high}$ = 27%, $PDL\text{-}1^{low}$ = 7% in two experiments with similar results).

Circulating $Ly6C^{low}CD11c^{+}$ monocytes containing apoptotic cells migrate into the spleen and express MHC II

Since $Ly6C^{low}$ monocytes can migrate into spleen and differentiate into splenic macrophages and DCs (15), we asked whether apoptotic cells can be transported into the spleen by monocytes and whether the monocyte phenotype changed in the spleen. Mice were injected with PKH67-labeled apoptotic cells and, 20 h later, when free apoptotic cells were no longer observed in blood as determined by immunofluorescence microscopy, blood from injected mice was pooled and adoptively transferred into a new recipient. The spleen sections of the new recipient were examined by confocal microscopy 40 h later. As shown in Fig. 2*D*, $PKH67^{+}$ cells, which were mostly $CD11c^{+}$ (*left panel*), could be observed in both the red pulp as well as in the T cell-rich area.

Although our results suggest that circulating $Ly6C^{low}$ monocytes can transport apoptotic cells into the spleen, the transfer of cellular contents between migrating monocytes and resident DCs may obscure their lineages (18,19). It remains possible that apoptotic cells found within the spleen of the monocyte recipients were cargo transferred either from other spleen DCs or from circulating monocytes. To determine whether apoptotic cell material was transferred between different cell populations, we performed a two-step adoptive transfer experiment exactly as above except that the first recipients were of the $CD45.1^{+}$ allotype and the second recipients had the CD45.2 allotype. At 48 h after this transfer, we isolated $CD11c^{+}$ cells from the spleen and examined the distribution of apoptotic cells. As shown in Fig. 3*A*, almost all apoptotic cells remained in the transferred $CD45.1^{+}CD11c^{+}CD8\alpha^{-}$ fraction. More importantly, unlike those in circulation and those from bone marrow, only apoptotic cell $(Apo)^{+}$ $CD11c^{+}CD8\alpha^{-}$ cells recovered from spleen expressed a significant amount of MHC II on their surface, suggesting that these cells differentiated in the splenic environment (Fig. 3*B*, *left*). Since a proportion of both Apo^{+} and $Apo^{-}CD11c^{+}$ mature monocytes recovered from the spleen expressed increased MHC II, ingestion of apoptotic cells did not impede monocyte differentiation (Fig. 3*B*, *right*). Confocal microscopy revealed that donor cells (CD45.1) containing apoptotic cells were present in both the marginal zones and T cell areas, and those colocalized with T cells acquired the morphology of DCs (Fig. 3*C*).

$Ly6C^{low}CD11c^{+}$ monocytes in spleen ingest apoptotic cells

Since the spleen is continuously being populated with monocytes from the circulation, and splenic monocytes share many phenotypic characteristics of their circulating counterparts (20), we next investigated baseline phenotypic markers of monocytes in the spleen. In CX3CR1/GFP mice, $CD11c^{int}$ mature monocytes could be distinguished from $CD11c^{high}$ DCs based on lower expression of CD11c and GFP, although GFP expression was more heterogeneous on DCs (Fig. 4*A*). Unlike $CD11c^{int}$ mature monocytes in the circulation, those in the spleen showed higher levels of expression of MHC II on their surface, suggesting that they were undergoing differentiation in the splenic environment (Fig. 4*B*). Further phenotypic

analysis revealed that most of $CD11c^{int}$ mature monocytes do not express typical DC markers on their surface, and their morphology was similar to circulating monocytes (Fig. 4*C* and data not shown). To further clarify the relationship between $CD11c^{int}GFP^{+}$ monocytes and $CD11c^{high}GFP^{+}$ DCs in the spleen, we depleted $Ly6C^{high}$ monocytes from PBLs using immunomagnetic sorting (15) and transferred the remaining $CD45.2^{+}Ly6C^{low}GFP^{+}$ monocytes into a CD45.1 host. As shown in Fig. 4*D*, a significant percentage of transferred monocytes recovered from the recipient spleen up-regulated their expression of CD11c, demonstrating that at least some $CD11c^{high}$ GFP^{+} DCs in the spleen were monocyte derived.

To examine the phenotypic properties of the monocytic and DC populations in the spleen that ingest circulating apoptotic cells, we injected CM-Dil (red dye)-labeled apoptotic cells into CX3CR1/GFP mice and examined their distribution at different time points. As shown in Fig. 4*E*, ~25% of apoptotic cells were detected in GFP^{high} cells at 20 h, and this percentage increased to ~33% at 40 h postinjection. Among phagocytes that ingested apoptotic cells, 19% were $CD11c^{int}GFP^{high}$ monocytes. Interestingly, most of $CD11c^{high}$ DCs containing apoptotic cells also expressed high-level GFP, and their percentage increased from 15% to 35% (Fig. 4*E*). The majority of these cells are $CD8\alpha^{-}$(Fig. 4*E*, *right panels*). When the distribution of apoptotic cells was examined by immunofluorescence microscopy, most apoptotic cell-containing GFP^{+} cells were located around the marginal zones, and some could be found in white pulp area (Fig. 4*F*). Consistent with the analysis by flow cytometry (Fig. 4*E*, R2), many apoptotic cells could be found in GFP^{-} cells, which mainly consist of macrophages.

Temporal changes in apoptotic cells distribution among monocytes and DCs in the spleen

To exclude any possible artifacts relating to CX3CR1 or GFP expression in the transgenic mice (21), we further examined apoptotic cell distribution among monocytes and DCs by injection of apoptotic cells into wild-type B6 mice. Because the traditional method of DC purification using density gradient enriches only DCs (22), we isolated both $CD11c^{int}$ monocytes and $CD11c^{high}$ DCs by positive selection. As shown in Fig. 5*A*, although PKH67-labeled apoptotic cells were mainly confined to the $CD11c^{+}$ $CD8\alpha^{+}$ subpopulation at 18 h after i.v. injection, most apoptotic cells were found in $CD11c^{+}CD8\alpha^{-}$ cells at 40 h after injection. To determine whether apoptotic cell containing $CD8\alpha^{+}$ and $CD8\alpha^{-}$ cells share similar expression of B7 family members as observed for monocytes described above, costimulatory and coinhibitory molecule expression on these two subsets were compared. Whereas B7-1, B7-2, and PDL-2 expression was similar, apoptotic cell-containing $CD8\alpha^{-}$cells expressed 5- to 6-fold more PDL-1 than did $CD8\alpha^{+}$ cells and other $CD11c^{-}CD11b^{+}$ APCs in the spleen, consistent with the phenotype of $CD11c^{+}$ monocytes described above (Fig. 2*B* and data not shown).

As shown above, the apoptotic cell-containing monocytes recovered from spleen do not express $CD8\alpha$ (Fig. 3). We further examined the expression of additional $CD8\alpha^{-}$ DC markers such as 33D1 and MHC II on apoptotic cell-containing $CD11c^{+}CD8\alpha^{-}$ cells (8). As shown in Fig. 5*C*, at 18 h after ingestion, 33D1 expression on $Apo^{+}CD11c^{+}CD8\alpha^{-}$ cells was very low, but the percentage of $33D1^{+}$ cells increased significantly at 40 h postinjection. A similar increase of MHC II^{+} cells was also observed. During the 18- to 40-h period, the expression level of PDL-1 by $Apo^{+}CD11c^{+}CD8\alpha^{-}$ cells remained constant. The increased expression of 33D1 and MHC II expression between 18 and 40 h postinjection are consistent with the observation that $CD11c^{+}CD8\alpha^{-}$ $PDL\text{-}1^{high}33D1^{-}$MHC II^{-} monocytes undergo differentiation in spleen. Because of the continuous influx of monocytes into the spleen, the temporal changes we observed likely reflect the contribution from both resident and circulating monocytes.

Ly6C^{low} monocytes cross-present apoptotic cell-associated Ag (OVA) and suppress the response of endogenous OVA-specific CD8 T cells

The established ability of CD11c^{+}CD8α^{+} resident splenic DCs to cross-present cell-associated Ag can be attributed to their high efficiency in ingestion of apoptotic cells (10,23). Since we demonstrated that Ly6C^{low} monocytes have the ability to phagocytose apoptotic cells, we examined their ability to directly cross-present OVA Ag to T cells. As shown in Fig. 6*A*, sorted circulating Ly6C^{low} monocytes were able to cross-present both apoptotic cell-associated OVA and soluble OVA protein. To determine whether apoptotic cells containing monocytes can tolerize endogenous T cell responses, we injected OVA- or BSA-infused apoptotic cells and collected PBLs from five mice at 24 h postinjection. We transferred pooled PBLs into a new host and challenged it with CFA-OVA 1 wk later. At 1 wk following CFA-OVA challenge, we restimulated spleen cells with OVA peptide (257–264) and used intracellular staining of IFN-γ to quantify responding OVA-specific CD8 T cells. As shown in Fig. 6, *B* and *C*, the number of OVA peptide-reactive CD8 T cells was 4- to 5-fold lower in mice that received apoptotic cell-containing PBLs compared with the control group. A similar reduction in response to OVA was observed in T cells isolated from draining lymph nodes (not shown). However, the suppression of CD8 T cell response by apoptotic cell-containing PBLs was not observed in the absence of PDL-1 (Fig. 6*C*), demonstrating the critical role of PDL-1 in monocyte-mediated T cell tolerance.

Discussion

On a quantitative basis, macrophages likely constitute the dominant cell responsible for removal of dying cells in vivo (1). However, the ability of macrophages to induce T cell tolerance has been questioned because of the qualitatively different Ag processing in macrophages as compared with DCs. In contrast, engulfment of apoptotic cells by immature DCs leads to presentation of peptides derived from the dying cells (24,25), and in vivo experiments clearly implicate DCs in CD8 T cell tolerance (26). The role of monocytes in this context has not been critically examined. In the present study, we report for the first time that Ly6C^{low} monocytes express high levels of PDL-1, ingest apoptotic cells, and continuously differentiate into PDL-1^{high}CD8α^{-} MHC II^{+} DCs in the spleen. These apoptotic cell-containing APCs have the potential to tolerize T cell responses.

A recent study found that both Ly6C^{high} and Ly6C^{low} monocyte subsets could phagocytose fluorescent nanoparticles, but ingestion of cell debris was not examined (20). The ability of Ly6C^{low} monocytes to ingest apoptotic cells as observed here is more consistent with the predominant labeling of Ly6C^{low} monocytes by latex beads (27,28). Whether similar ligands and receptors described for macrophages (1) are utilized for ingestion of apoptotic cells by monocytes remains to be determined. It was proposed that the patrolling behavior of CXCR3^{high}Ly6C^{low} monocytes may give them more opportunity to interact with dying cells (29). However, we did not observe any alteration of apoptotic cell uptake by circulating monocytes in CD18$^{-/-}$ mice (our unpublished observations) in which the tethering of Ly6C monocyte is abolished. The tethering of monocytes along blood vessels is also not likely to be required for ingestion, as non-adherent isolated Ly6C^{low} monocytes remain highly efficient in the phagocytosis of apoptotic cells as shown here.

The differentiation of monocytes to DCs (migratory DCs) was observed some time ago (14); however, only recently have the distinct functional properties of monocyte subsets been appreciated. Ly6C^{high}CCR2^{+} monocytes accumulate rapidly in the peritoneal cavity after injection of thioglycolate (15). These monocytes up-regulate their expression of CD11c and MHC II and may be functionally equivalent to TNF-inducible NO synthase-producing DCs (Tip-DCs) that are also derived from monocytes (30). How Tip-DCs modulate T cell function remains controversial (30,31). In contrast, Ly6C^{low}CCR2^{-} monocytes do not show appreciable

accumulation or change in CD11c expression in response to inflammation. The limited response of circulating Ly6C^{low} monocytes to inflammatory stimuli was further evident from their lack of response to LPS stimulation. Unlike Ly6C^{high} monocytes that quickly disappeared from the blood within 20 h, the percentage of Ly6C^{low} monocytes was stable and the uptake of apoptotic cells not reduced. Furthermore, apoptotic cell-containing monocytes failed to up-regulate MHC II in the spleen after LPS treatment (our unpublished observation). Similarly, Balazs et al. (32) reported that a significant number of CD11c^{int} circulating blood DCs ingest fluorescently labeled bacteria, travel to the spleen, and contribute to marginal zone B cell response. It is noteworthy that the CD11c^{int} blood DCs do not express MHC II during the course of infection, suggesting that they have not yet fully differentiated into classic CD11c^{high} MHC II DCs and are likely to remain CD11c^{int} Ly6C^{low} monocytes. The signals that regulate monocyte/DC differentiation may also differ under steady-state vs inflammatory conditions. Unlike inflammatory Ly6C^{high} monocytes, the differentiation of Ly6C^{low} monocytes to DCs in the spleen in the steady-state was not affected by CCR7 deficiency (our unpublished observation).

To what extent Ly6C^{low} monocytes contribute to the splenic DC pool may depend on the context. In unmanipulated hosts, it has been shown that most CD8α^+ and CD8α^- DCs arise from a splenic pre-conventional DC precursor and that Ly6C^{low} monocytes contribute to only a small fraction of splenic DCs (12,33). In hosts that have undergone significant alterations in splenic composition, for example by irradiation, contribution by monocytes can be more substantial (34). Although the relationships between monocytes and DCs need further clarification, they also suggest that the splenic environment can influence monocyte/DC differentiation. Consistent with this notion, we observed that monocytes containing apoptotic cells up-regulate MHC II expression in the spleen but not in the bone marrow. Interestingly, almost all splenic DCs that were derived from apoptotic cell-containing monocytes were CD8α^-. Whereas resident CD11c$^+$CD8α^+ splenic DCs are known for their high efficiency in uptake of apoptotic cells (Refs. 10,23 and our unpublished observation), most CD11c^{high} CX3Cr1^{high} DC-containing apoptotic cells did not express CD8α (Fig. 4*E*). Because CD11c^{high}CX3CR1^{high} DCs can be directly derived from monocytes (Fig. 4*D*), we propose that this population represents the newly differentiated subset from monocytes and that CX3CR1 expression is down-regulated as the DCs mature. Our findings imply that the functional boundaries between monocytes, macrophages, and DCs are less distinct in peripheral tissue, as was also implied by variable phenotypic and functional properties of monocyte-derived DCs in the lung and skin (35).

Although in vitro observations and analysis of DCs following certain virus infections have suggested that apoptotic cells can be transferred from one DC to a neighboring one (19), we did not observe exchange of apoptotic material between adoptively transferred monocytes/DCs and the endogenous resident DCs. The ability of Ly6C^{low} monocytes and monocyte-derived DCs to directly cross-present cell-associated Ag to CD8 T cells, the lack of transfer of apoptotic material to resident DCs, and the migration of monocyte-derived DCs to the T cell area in the spleen strongly suggest that monocyte-derived DCs can directly interact with T cells. The direct presentation of Ag by monocyte-derived DCs to CD8$^+$ T cells in the skin during immunization has also been observed (36). Thus, it appears that DCs derived from Ly6C^{high} and Ly6C^{low} monocytes are divided in their functions, with the former providing productive immune responses and the latter able to play a role in immune tolerance. Furthermore, it is obvious that T cell tolerance is achieved through the activity of multiple different types of APCs.

The Ly6C^{low} monocyte subset that ingested apoptotic cells expressed high levels of PDL-1, a phenotype characteristic that was maintained following their migration to the spleen. Despite the fact that PDL-1-deficient mice on normal strain backgrounds do not develop overt autoimmunity, a pivotal role of PDL-1 in maintaining peripheral tolerance has been

demonstrated in PDL-1-deficient autoimmune NOD mice (37,38). In these mice, increased numbers of autoreactive T cells escape to the periphery (39). We observed that, in the absence of PDL-1, monocyte-containing apoptotic cells failed to suppress endogenous OVA-specific CD8 T cell responses. Whether the expression of PDL-1 is required to maintain regulatory CD4 T cell functions or to directly anergize/delete CD8 T cells remains to be addressed.

Expansion of different monocyte subsets has been observed in various chronic inflammatory and autoimmune diseases (40–42). Recruitment of monocytes during acute injury can facilitate the resolution of tissue damage (20,43). The ability of the $Ly6C^{low}$ monocyte subset to engulf dying cell debris and to suppress T cell response demonstrates its potential role in maintaining homeostasis of both innate and adaptive immunity. Whether apoptosis induced by virus infections such as HIV (44,45) can subvert immune responses and induce tolerance through the mechanism described herein remains to be determined.

Acknowledgments

We thank Dr. Cong-Qui Chu for his critical reading of the manuscript and Dr. Gwendolyn Randolph for advice.

References

1. Savill J, Dransfield I, Gregory C, Haslett C. A blast from the past: clearance of apoptotic cells regulates immune responses. Nat Rev Immunol 2002;2:965–975. [PubMed: 12461569]
2. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. Annu Rev Immunol 2003;21:685–711. [PubMed: 12615891]
3. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-β, PGE_2, and PAF. J Clin Invest 1998;101:890–898. [PubMed: 9466984]
4. Kim S, Elkon KB, Ma X. Transcriptional suppression of interleukin-12 gene expression following phagocytosis of apoptotic cells. Immunity 2004;21:643–653. [PubMed: 15539151]
5. Cvetanovic M, Mitchell JE, Patel V, Avner BS, Su Y, van der Saag PT, Witte PL, Fiore S, Levine JS, Ucker DS. Specific recognition of apoptotic cells reveals a ubiquitous and unconventional innate immunity. J Biol Chem 2006;281:20055–20067. [PubMed: 16707494]
6. Steinman RM, Turley S, Mellman I, Inaba K. The induction of tolerance by dendritic cells that have captured apoptotic cells. J Exp Med 2000;191:411–416. [PubMed: 10662786]
7. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005;5:953–964. [PubMed: 16322748]
8. Dudziak D, Kamphorst AO, Heidkamp GF, Buchholz VR, Trumpfheller C, Yamazaki S, Cheong C, Liu K, Lee HW, Park CG, et al. Differential antigen processing by dendritic cell subsets in vivo. Science 2007;315:107–111. [PubMed: 17204652]
9. Maldonado-Lopez R, De Smedt T, Michel P, Godfroid J, Pajak B, Heirman C, Thielemans K, Leo O, Urbain J, Moser M. $CD8\alpha^+$ and $CD8\alpha^-$ subclasses of dendritic cells direct the development of distinct T helper cells in vivo. J Exp Med 1999;189:587–592. [PubMed: 9927520]
10. Liu K, Iyoda T, Saternus M, Kimura Y, Inaba K, Steinman RM. Immune tolerance after delivery of dying cells to dendritic cells in situ. J Exp Med 2002;196:1091–1097. [PubMed: 12391020]
11. Iyoda T, Shimoyama S, Liu K, Omatsu Y, Akiyama Y, Maeda Y, Takahara K, Steinman RM, Inaba K. The $CD8^+$ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. J Exp Med 2002;195:1289–1302. [PubMed: 12021309]
12. Naik SH, Metcalf D, van Nieuwenhuijze A, Wicks I, Wu L, O'Keeffe M, Shortman K. Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. Nat Immunol 2006;7:663–671. [PubMed: 16680143]
13. Fogg DK, Sibon C, Miled C, Jung S, Aucouturier P, Littman DR, Cumano A, Geissmann F. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. Science 2006;311:83–87. [PubMed: 16322423]

14. Randolph GJ, Inaba K, Robbiani DF, Steinman RM, Muller WA. Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. Immunity 1999;11:753–761. [PubMed: 10626897]
15. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity 2003;19:71–82. [PubMed: 12871640]
16. Latchman YE, Liang SC, Wu Y, Chernova T, Sobel RA, Klemm M, Kuchroo VK, Freeman GJ, Sharpe AH. PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells. Proc Natl Acad Sci USA 2004;101:10691–10696. [PubMed: 15249675]
17. Carbone FR, Bevan MJ. Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. J Exp Med 1990;171:377–387. [PubMed: 2137512]
18. Allan RS, Waithman J, Bedoui S, Jones CM, Villadangos JA, Zhan Y, Lew AM, Shortman K, Heath WR, Carbone FR. Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. Immunity 2006;25:153–162. [PubMed: 16860764]
19. Carbone FR, Belz GT, Heath WR. Transfer of antigen between migrating and lymph node-resident DCs in peripheral T-cell tolerance and immunity. Trends Immunol 2004;25:655–658. [PubMed: 15530835]
20. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, Libby P, Weissleder R, Pittet MJ. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med 2007;204:3037–3047. [PubMed: 18025128]
21. Jakubzick C, Tacke F, Ginhoux F, Wagers AJ, van Rooijen N, Mack M, Merad M, Randolph GJ. Blood monocyte subsets differentially give rise to $CD103^{+}$ and $CD103^{-}$ pulmonary dendritic cell populations. J Immunol 2008;180:3019–3027. [PubMed: 18292524]
22. Inaba K, Turley S, Yamaide F, Iyoda T, Mahnke K, Inaba M, Pack M, Subklewe M, Sauter B, Sheff D, et al. Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. J Exp Med 1998;188:2163–2173. [PubMed: 9841929]
23. Schulz O, Reis e Sousa C. Cross-presentation of cell-associated antigens by $CD8\alpha^{+}$ dendritic cells is attributable to their ability to internalize dead cells. Immunology 2002;107:183–189. [PubMed: 12383197]
24. Hawiger D, Inaba K, Dorsett Y, Guo M, Mahnke K, Rivera M, Ravetch JV, Steinman RM, Nussenzweig MC. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J Exp Med 2001;194:769–779. [PubMed: 11560993]
25. Roncarolo MG, Levings MK, Traversari C. Differentiation of T regulatory cells by immature dendritic cells. J Exp Med 2001;193:F5–9. [PubMed: 11208869]
26. Redmond WL, Sherman LA. Peripheral tolerance of CD8 T lymphocytes. Immunity 2005;22:275–284. [PubMed: 15780985]
27. Tacke F, Ginhoux F, Jakubzick C, van Rooijen N, Merad M, Randolph GJ. Immature monocytes acquire antigens from other cells in the bone marrow and present them to T cells after maturing in the periphery. J Exp Med 2006;203:583–597. [PubMed: 16492803]
28. Sunderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, Leenen PJ. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. J Immunol 2004;172:4410–4417. [PubMed: 15034056]
29. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, Sarnacki S, Cumano A, Lauvau G, Geissmann F. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science 2007;317:666–670. [PubMed: 17673663]
30. Serbina NV, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. Immunity 2003;19:59–70. [PubMed: 12871639]
31. Geissmann F, Auffray C, Palframan R, Wirrig C, Ciocca A, Campisi L, Narni-Mancinelli E, Lauvau G. Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses. Immunol Cell Biol 2008;86:398–408. [PubMed: 18392044]
32. Balazs M, Martin F, Zhou T, Kearney J. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. Immunity 2002;17:341–352. [PubMed: 12354386]

33. Varol C, Landsman L, Fogg DK, Greenshtein L, Gildor B, Margalit R, Kalchenko V, Geissmann F, Jung S. Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. J Exp Med 2007;204:171–180. [PubMed: 17190836]
34. Leon B, Martinez del Hoyo G, Parrillas V, Vargas HH, Sanchez-Mateos P, Longo N, Lopez-Bravo M, Ardavin C. Dendritic cell differentiation potential of mouse monocytes: monocytes represent immediate precursors of $CD8^-$ and $CD8^+$ splenic dendritic cells. Blood 2004;103:2668–2676. [PubMed: 14630812]
35. Klechevsky E, Morita R, Liu M, Cao Y, Coquery S, Thompson-Snipes L, Briere F, Chaussabel D, Zurawski G, Palucka AK, et al. Functional specializations of human epidermal Langerhans cells and $CD14^+$ dermal dendritic cells. Immunity 2008;29:497–510. [PubMed: 18789730]
36. Le Borgne M, Etchart N, Goubier A, Lira SA, Sirard JC, van Rooijen N, Caux C, Ait-Yahia S, Vicari A, Kaiserlian D, Dubois B. Dendritic cells rapidly recruited into epithelial tissues via CCR6/CCL20 are responsible for $CD8^+$ T cell crosspriming in vivo. Immunity 2006;24:191–201. [PubMed: 16473831]
37. Ansari MJ, Salama AD, Chitnis T, Smith RN, Yagita H, Akiba H, Yamazaki T, Azuma M, Iwai H, Khoury SJ, et al. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. J Exp Med 2003;198:63–69. [PubMed: 12847137]
38. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, Koulmanda M, Freeman GJ, Sayegh MH, Sharpe AH. Tissue expression of PD-L1 mediates peripheral T cell tolerance. J Exp Med 2006;203:883–895. [PubMed: 16606670]
39. Anderson MS, Bluestone JA. The NOD mouse: a model of immune dysregulation. Annu Rev Immunol 2005;23:447–485. [PubMed: 15771578]
40. Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, Pittet MJ. Ly-$6C^{hi}$ monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. J Clin Invest 2007;117:195–205. [PubMed: 17200719]
41. Nikolic T, Bouma G, Drexhage HA, Leenen PJ. Diabetes-prone NOD mice show an expanded subpopulation of mature circulating monocytes, which preferentially develop into macrophage-like cells in vitro. J Leukocyte Biol 2005;78:70–79. [PubMed: 15788439]
42. Amano H, Amano E, Santiago-Raber ML, Moll T, Martinez-Soria E, Fossati-Jimack L, Iwamoto M, Rozzo SJ, Kotzin BL, Izui S. Selective expansion of a monocyte subset expressing the CD11c dendritic cell marker in the Yaa model of systemic lupus erythematosus. Arthritis Rheum 2005;52:2790–2798. [PubMed: 16142734]
43. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol 2008;8:349–361. [PubMed: 18437155]
44. Lewis DE, Tang DS, Adu-Oppong A, Schober W, Rodgers JR. Anergy and apoptosis in $CD8^+$ T cells from HIV-infected persons. J Immunol 1994;153:412–420. [PubMed: 7515928]
45. McCloskey TW, Bakshi S, Than S, Arman P, Pahwa S. Immunophenotypic analysis of peripheral blood mononuclear cells undergoing in vitro apoptosis after isolation from human immunodeficiency virus-infected children. Blood 1998;92:4230–4237. [PubMed: 9834228]

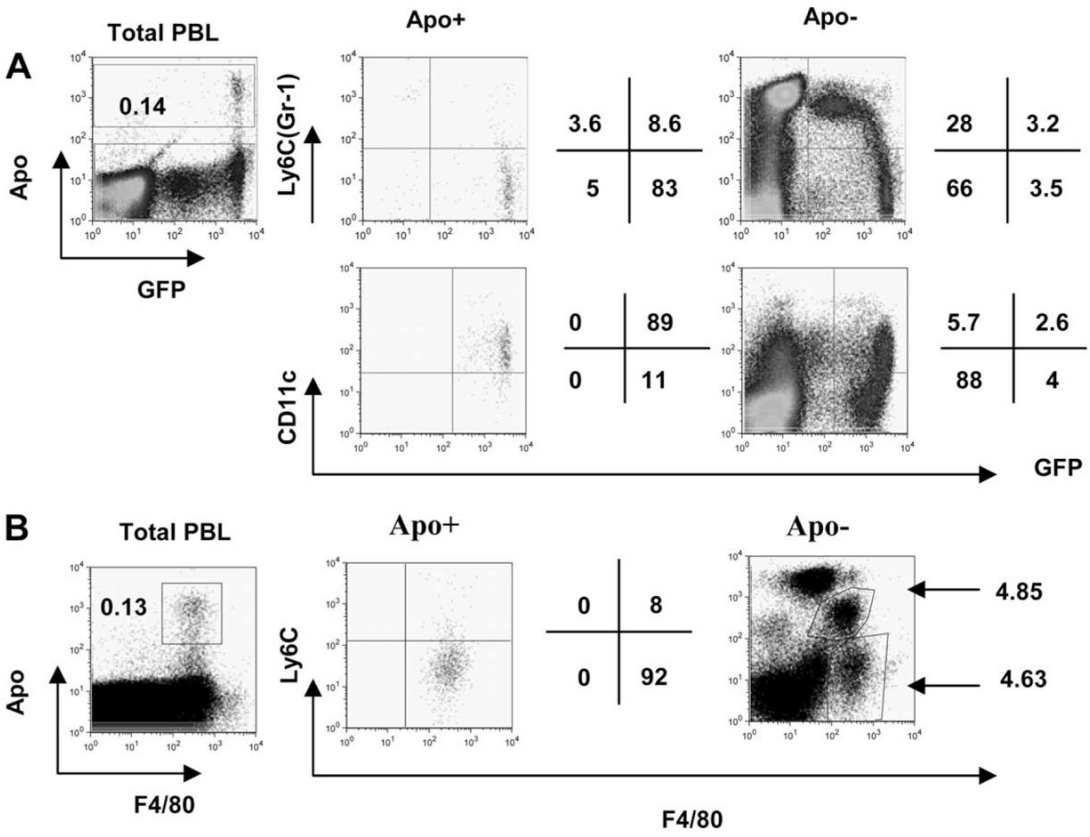

FIGURE 1.

Circulating Ly6C^{low} mature monocytes ingest apoptotic cells. CM-Dil-labeled apoptotic thymocytes (30×10^6) were injected i.v. into either CX3CR1/GFP (*A*) or wild-type B6 mice (*B*). Circulating leukocytes that did (Apo$^+$) or did not (Apo$^-$) contain apoptotic cells were analyzed for their expression of GFP, Ly6C (Gr-1) (*A*, *upper panel*), CD11c (*A*, *lower panel*), and F4/80, Ly6C (*B*). Similar results were obtained in five independent experiments.

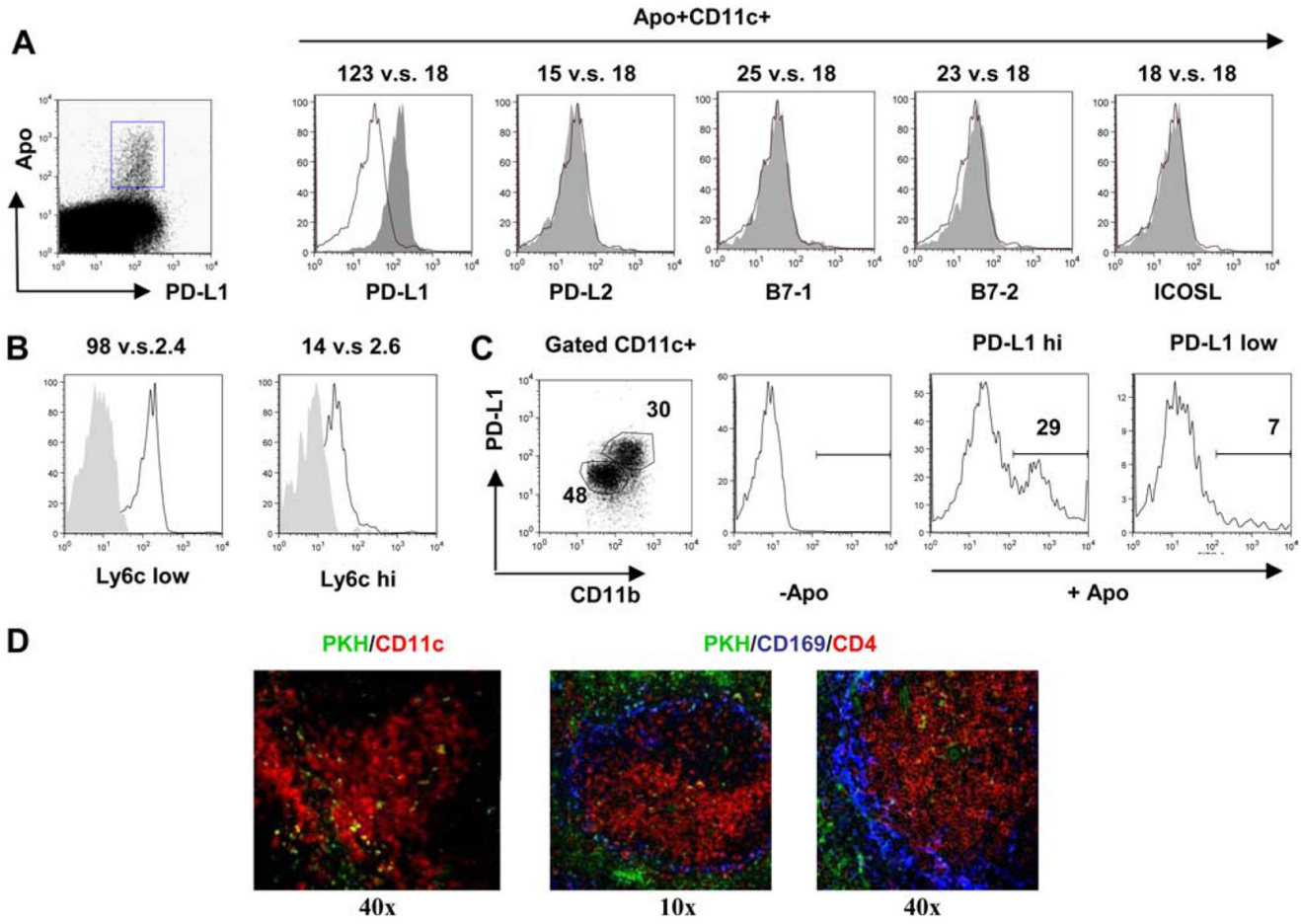

FIGURE 2.

Circulating monocytes containing apoptotic cells express high levels of PDL-1 and migrate into the spleen. *A*, PKH67-labeled apoptotic cells (30×10^6) were injected as in Fig. 1 and mouse peripheral blood samples were collected at 20 h after injection. $PKH67^+$ $CD11c^+$ cells were further analyzed for their expression of B7 family members (open histogram shows isotype control). Results are representative of five experiments. *B*, Peripheral monocytes were analyzed for their expression of PDL-1 (gray histogram shows isotype control). *C*, Peripheral blood from three mice was collected and $CD11c^+CD11b^+$ cells were sorted based on their PDL-1 expression. PDL-1^{low} and PDL-1^{high} fractions were incubated with PKH67-labeled apoptotic cells for 4 h, and uptake of apoptotic cells was analyzed by flow cytometry. The result is representative of two experiments. *D*, PKH67-labeled apoptotic cells were injected i.v. as in Fig. 1 into five recipients. Peripheral blood was collected after 20 h, pooled, and transferred into a new recipient. Forty hours postinjection, the recipient spleen was harvested and frozen sections were stained for CD11c (*left panel*, ×40x), CD4 (T cell), CD169 (sialoadhesin-positive marginal zone macrophages) (*middle panel*, ×10; *right panel*, ×40). Similar results were obtained from two experiments.

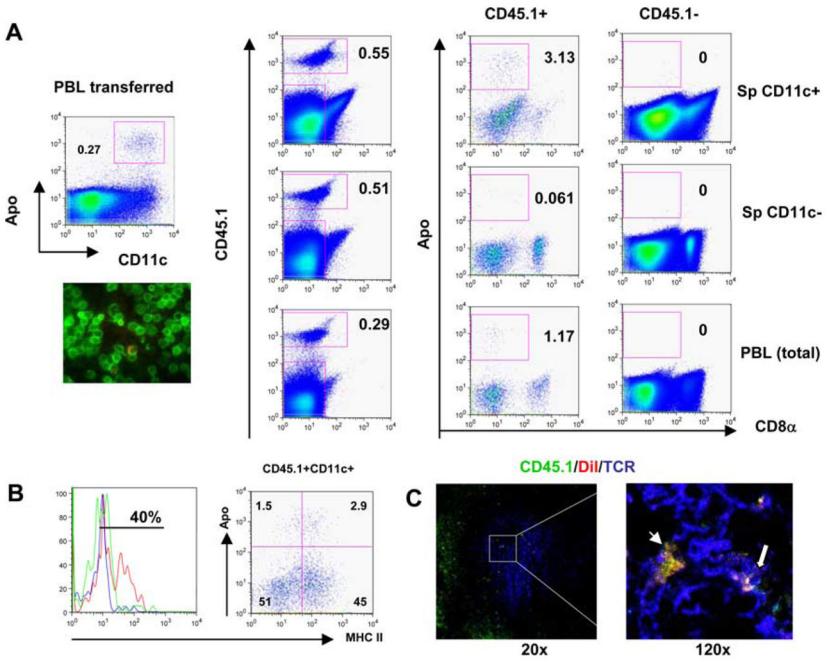

FIGURE 3.

Circulating apoptotic cell-containing Ly6C^{low} monocytes differentiate into DCs in the spleen. *A*, CM-Dil red apoptotic cells were injected into CD45.1 allotype recipients and analyzed at 20 h as in Fig. 2. The percentage of CD11c$^+$ monocytes that had ingested apoptotic cells were analyzed by flow cytometry (*left upper panel*) and by immunofluorescence microscopy (*left lower panel*: CD45.1, green; CM-Dil, red). Cells were transferred into a secondary (CD45.2) recipient, and at 40 h after transfer, CD11c$^+$ cells from spleen were isolated. The presence of apoptotic cells was analyzed by flow cytometry in both the CD11c$^+$ and CD11c$^-$ fractions as well as total PBLs. The CD45.1 congenic marker was used to distinguished monocytes from donor vs recipient. Note that apoptotic cells were confined within the CD45.1$^+$CD11c$^+$ fraction, demonstrating that transfer of apoptotic cell material between transferred monocytes and endogenous DCs is minimal. *B*, *Left*, CD45.1$^+$CD11c$^+$Apo$^+$ monocytes were recovered from the secondary recipient by CD11c-positive selection from the spleen (red), bone marrow (green), and the circulation (blue), and the expression of MHC II was analyzed by flow cytometry. *Right*, Expression of MHC II on transferred Apo$^+$ and Apo$^-$ CD45.1$^+$CD11c$^+$ cells recovered from spleen. The gate for MHC II expression was based on endogenous CD11c$^+$ cells. Results from *A* and *B* are representative of three experiments. *C*, Confocal microscopic analysis of adoptively transferred CD45.1 blood cells in the spleen of CD45.2 mice 40 h after transfer. *Left panel* (×20), Blood cells not containing apoptotic cells are green and are present mostly in the red pulp. CD45.1$^+$Apo$^+$ (yellow, see also arrowhead, *right panel*) cells were found in both marginal zone and T cell area. *Right panel* (×120), Direct contact between Apo-containing monocyte-derived DCs and T cells produces a white color (white arrow) (CD45.1, green; Apo$^+$, red; T cells, blue).

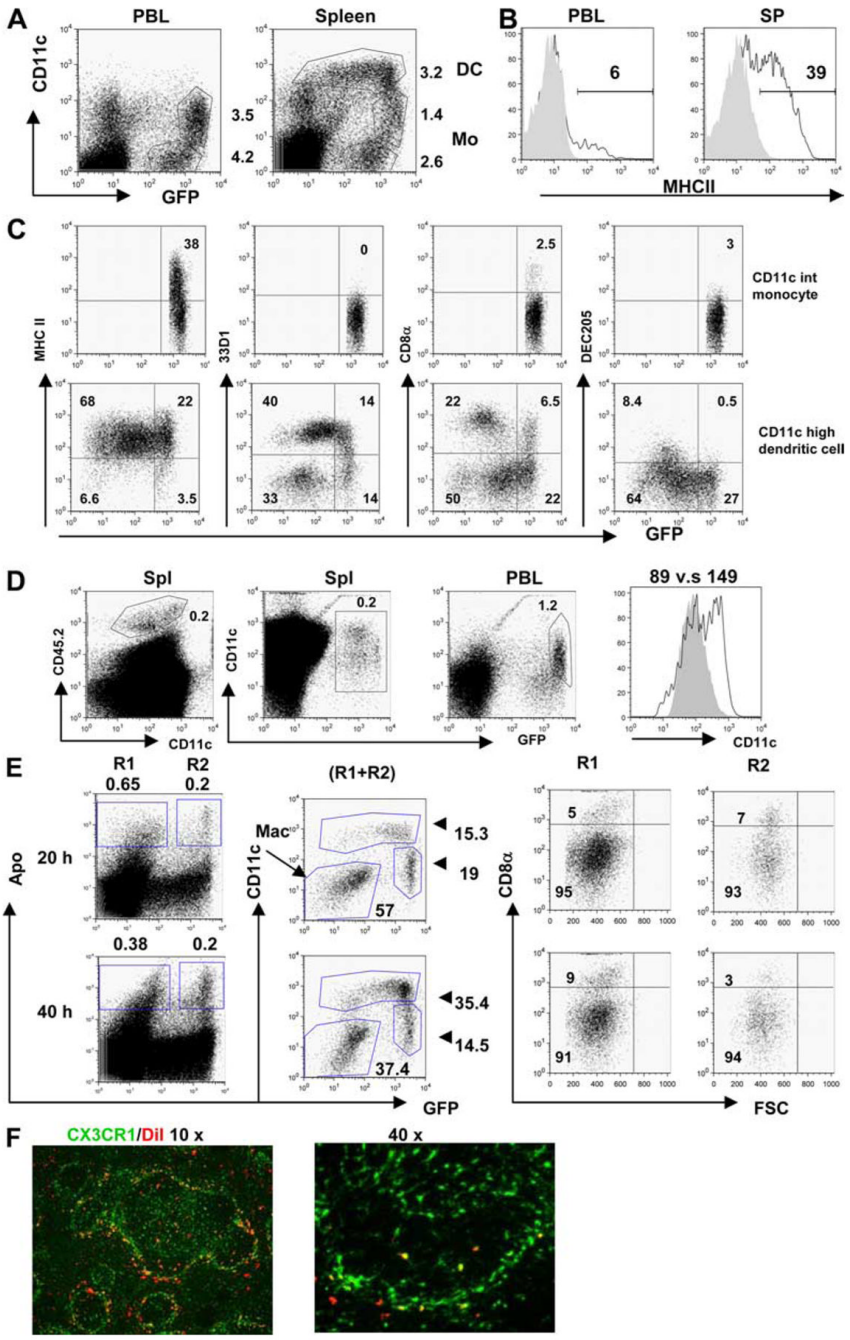

FIGURE 4.

Monocytes in spleen ingest apoptotic cells. *A*, CX3CR1/GFP expression by splenic $CD11c^{-}CD11c^{int}$ monocytes and $CD11c^{high}$ DCs. The profile of blood monocyte was included for comparison. *B*, Comparison of MHC II expression on $CD11c^{int}CX3CR1/GFP^{high}$ mature monocytes from peripheral blood and spleen. *C*, Expression of DC markers on $CD11^{int}$ monocytes (*upper panels*) and $CD11c^{high}$ DCs (*lower panels*) in spleen. *D*, Pooled PBLs from five $CD45.2^{+}CX3CR1/GFP^{+/-}$ mice were depleted of $Ly6C^{high}$ population using anti-Ly6C beads and transferred into a $CD45.1^{+}$ host. $CD11c^{+}$ spleen cells were isolated 2 days later from the recipient, and the phenotypes of transferred monocytes were analyzed by flow cytometry. For comparison, the CD11c expression on peripheral monocytes (filled histogram) was

overlaid with those recovered from spleen (open histogram). Results are representative of two experiments. *E*, CM-Dil (PE)-labeled apoptotic thymocytes were injected i.v. into CX3CR1/GFP mice as in Fig. 1. Spleen cells that ingested apoptotic cells were gated as GFP^{-}(R1) or GFP^{+} (R2) and their expression of CD11c and CD8α was analyzed by flow cytometry at 20 and 40 h postinjection. Results are representative of two experiments. *F*, Spleen sections of CX3CR1/GF) mice showing the distribution of apoptotic cells (CM-Dil red) by immunofluorescence microscopy at 40 h after injection.

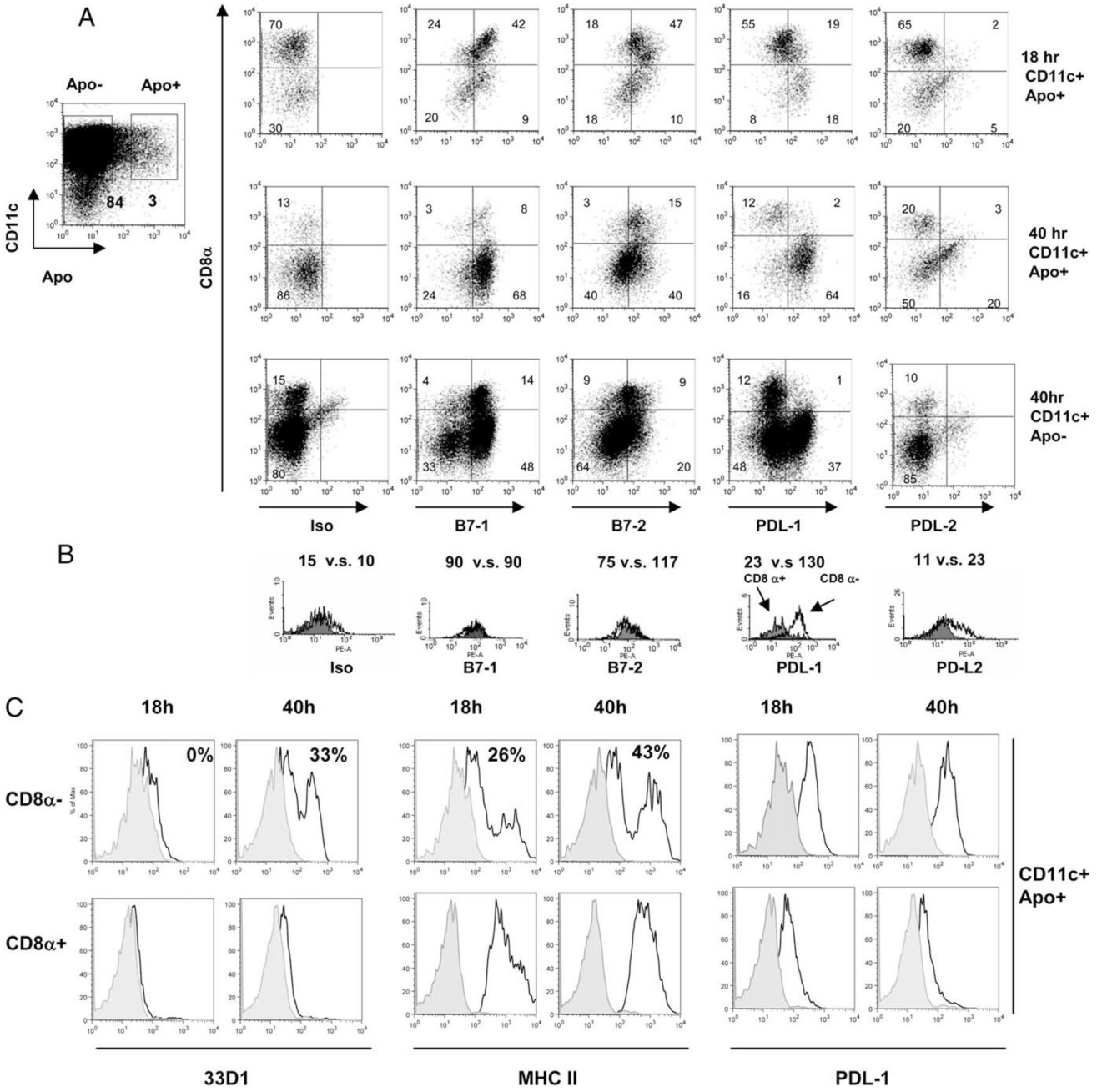

FIGURE 5.

Temporal distribution of apoptotic cells among monocytes and DCs in spleen. *A*, PKH67 (FITC)-labeled apoptotic thymocytes were injected i.v. into B6 mice, and the $CD11c^+$ splenic monocytes and DCs were purified by positive selection at either 18 (*upper panel*) or 40 h (*lower two panels*) after injection. Different $CD11c^+$ subsets were distinguished by their CD8*α* expression (*y*-axis). The expression of B7-1, B7-2, PDL-1, and PDL-2 (*x*-axis) on $CD11c^+PKH67^+$ (containing apoptotic cells) and $CD11c^+PKH67^-$ were analyzed by flow cytometry. *B*, The expression of B7 family molecules on $Apo^+CD8\alpha^+$(filled histogram) and $Apo^+CD8\alpha^-$(open histogram) cells are overlaid and their geometric mean fluorescence intensities are shown ($CD8\alpha^+$ vs $CD8\alpha^-$). The results are representative of five experiments.

C, The expression of 33D1, MHC II, and PDL-1 (open histograms) on each subset was analyzed by flow cytometry. Filled histograms show isotype controls. Results are representative of two experiments.

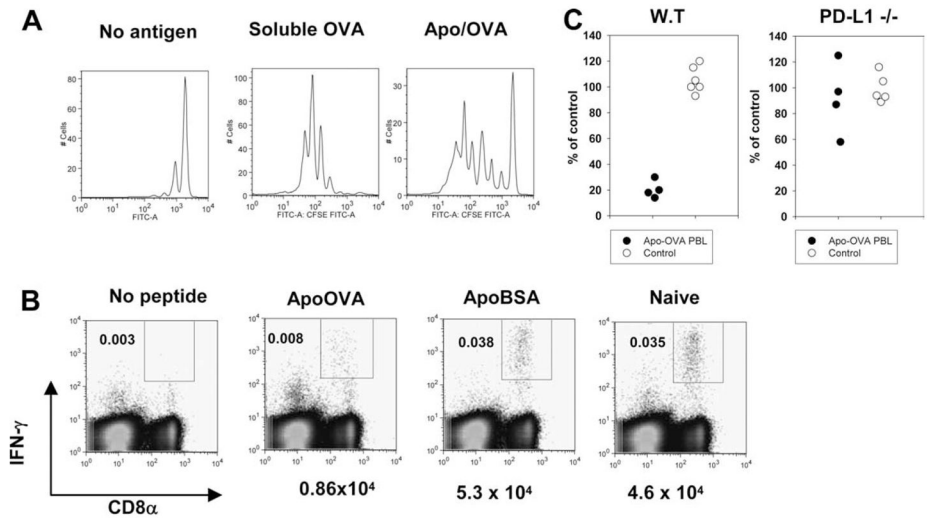

FIGURE 6.

Ly6C^{low} monocytes can directly cross-present apoptotic cell-associated Ags and suppress endogenous Ag-specific T cell response through PDL-1. *A*, Ly6C^{low} monocytes (2×10^5) were sorted by their CD11c and PDL-1 expression from pooled blood of five mice and incubated with either 100 μg of soluble OVA or 1×10^6 OVA-containing apoptotic cells. CFSE-labeled OT-I cells (1×10^6) were added to the culture and CFSE dilution was analyzed by flow cytometry 3 days later. *B*, OVA- or BSA-loaded apoptotic cells (30×10^6) were injected i.v. into five recipients and, after 24 h, pooled PBLs were transferred into a new host. One week after transfer into the secondary recipients, the recipients and control naive mice were challenged with CFA-OVA s.c. One week after the challenge, spleen cells were collected and restimulated with OVA peptide. The percentage and absolute number (below *x*-axis) of IFN-γ-producing CD8 T cells from each host was analyzed by flow cytometry. *C*, Summary of three independent experiments in wild-type and PDL-1-deficient mice. Each mouse in Apo-OVA PBL group received pooled PBLs from five mice. In each experiment, two to three naive mice challenged with CFA-OVA were used as controls and their average response was set as 100%.