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Type I IFNs Control Antigen Retention and Survival of CD8α+ Dendritic Cells after Uptake of Tumor Apoptotic Cells Leading to Cross-Priming

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Cross-presentation is a crucial mechanism for generating CD8 T cell responses against exogenous Ags, such as dead cell-derived Ag, and is mainly fulfilled by CD8α+ dendritic cells (DC). Apoptotic cell death occurring in steady-state conditions is largely tolerogenic, thus hampering the onset of effector CD8 T cell responses. Type I IFNs (IFN-I) have been shown to promote cross-presentation of CD8 T cells against soluble or viral Ags, partly through stimulation of DC. By using UV-irradiated OVA-expressing mouse EG7 thymoma cells, we show that IFN-I promote intracellular Ag persistence in CD8α+ DC that have engulfed apoptotic EG7 cells, regulating intracellular pH, thus enhancing cross-presentation of apoptotic EG7-derived OVA Ag by CD8α+ DC. Notably, IFN-I also sustain the survival of Ag-bearing CD8α+ DC by selective upmodulation of antiapoptotic genes and stimulate the activation of cross-presenting DC. The ensemble of these effects results in the induction of CD8 T cell effector response in vitro and in vivo. Overall, our data indicate that IFN-I cross-prime CD8 T cells against apoptotic cell-derived Ag both by licensing DC and by enhancing cross-presentation. The Journal of Immunology, 2011, 186: 000–000.

Cross-presentation of cell-associated Ag, such as dead cell-derived Ag, is a crucial process for generating CD8 T cell responses to Ag that are not expressed by APC, such as viruses that do not infect APC or tumors of nonhematopoietic origin (1). Among APC, dendritic cells (DC) are specialized for cross-presentation, and accumulating literature indicates that in vivo this process is mainly fulfilled by the CD8α+ DC subset (2). In the steady state, CD8α+ DC constitutively cross-present self Ag, such as material derived from apoptotic cells as a result of constitutive cell turnover, leading to self-tolerance (3). However, in the context of infection or pathological distress, signals consisting of microbial compounds or of inflammatory stimuli released by cells of innate immunity act as danger signals that induce DC activation, in a process referred to as licensing of DC to cross-priming (4). The underlying molecular mechanisms that result from such licensing are still under investigation and include enhanced costimulatory signals and diminished proapoptotic signals by the DC (5). It has been reported that triggering of TLR4 increases the efficiency of peptide presentation on MHC-I molecules by DC, suggesting that the enhancement of cross-presentation may represent another mechanism promoting cross-presentation (6). With regard to the mechanisms regulating cross-presentation, it is known that presentation of particulate Ag, such as cellular Ag, is critically dependent on the timing of persistence within phagosomal compartment, a process governed by intraphagosomal pH (6). Studies from Amigorena laboratory have established a strict correlation between phagosomal alkalization, which delays the proteolytic activity of lysosomal enzymes, and the efficiency of cross-presentation (7).

Type I IFNs (IFN-I) are a family of inflammatory cytokines produced by innate cells upon pathogenic challenge, playing multiple roles in the stimulation of immune responses, including DC activation and CD8 T cell effector function in vitro and in vivo. Of interest, IFN-I have been shown to promote cross-priming against viral or protein Ag, partly through the stimulation of DC (8–10). Furthermore, cross-priming stimulated by TLR3, TLR4, and TLR9 ligands was shown to be critically dependent on IFN-I signaling, implying these cytokines as important mediators in infection-stimulated cross-priming (9). Recent work from our laboratory has shown that human DC generated in the presence of IFN-α exhibit enhanced cross-presentation of allogeneic apoptotic cell Ag to autologous CD8 T cells, suggesting a role for IFN-I in DC cross-presentation of cell-associated Ag (11). In this study, we demonstrate that IFN-I promote cross-priming in vivo against cell-associated Ag derived from tumor apoptotic cells through multiple actions on CD8α+ DC, as follows: 1) by enhancing Ag persistence and, thus, cross-presentation; 2) by sustaining the survival of Ag-bearing DC selectively; and 3) by activating DC.

Materials and Methods

**Mice**

Female C57BL/6 mice (5–7 wk old) and OT-I TCR-transgenic mice were purchased from Charles River Laboratories. IFN regulatory factor (IRF)-
8−/− mice were generated and bred, as described (12). All mice were manipulated in accordance with the local Ethical Committee guidelines.

Reagents

High-titer IFN-I was prepared from the C2A3-3 cell line, as described in detail elsewhere (13). Anti-mouse IFN-I sheep Ig was used at 1000 neutralizing units (14). For flow cytometry, the following mAbs were used: anti-CD11c, which was used either in FITC, PE, or allophycocyanin form; PE anti-CD8, CD25, and CD69; biotin anti-CD40, CD86, and I-A (all from BD Pharmingen); tricolor anti-CD8 (Caltag Laboratories); and biotin anti-mouse MHC class I molecule KK bound to the peptide SHINFER of OVA (MHCI; a gift from Dr. W. Cavanagh, clone 25-D1,16; eBioscience). Biotinylated mAbs were detected with streptavidin PerCP (BD Pharmingen) or streptavidin tricolor (Caltag Laboratories). Lysosensor green DND-189 (Invitrogen) was used at 5 μM. Diphenylethionon DDI (DPI; Sigma-Aldrich) was used at 5 μM.

Isolation of DC and OT-I lymphocytes

The procedure of splenic DC isolation has been described in detail elsewhere (15). Briefly, total splenocytes were subjected to density-gradient centrifugation in Nycodenz solution (1.077 g/ml; Life Technologies). The procedure of splenic DC isolation has been described in detail elsewhere (15). The DC subsets using FACSAria cell sorter (BD Biosciences), yielding 99% purity. CD8 T cells from OT-I splenocytes and lymph node (LN) cells were purified using anti-CD8 microbeads (Miltenyi Biotec). Purity routinely ranged: 95–98%.

Culture and apoptosis induction of EG.7-OVA cell line

The EG.7-OVA cell line (EG7, CRL-2113; American Type Culture Collection), a stable transfectant of the murine OVA-expressing EL4 thymoma (H-2b), was maintained in complete RPMI 1640 medium supplemented with 4148 (0.4 mg/ml; Calbiochem). For uptake assays, EG7 cells were labeled with the dye CFSE (1 μM; Invitrogen). Cells were then washed and resuspended in PBS/1% FCS at 6 × 10^6/ml and UV irradiated (λ = 254 nm) at a 9-cm distance for 3 min. Cells were then resuspended in IMDM complete medium (4 × 10^6 cells/ml) and incubated at 37°C, 5% CO2 overnight. Apoptosis was FACS analyzed by annexin V (Roche) and propidium iodide (PI; Sigma-Aldrich) staining (data not shown).

Assays for apoptotic cell uptake

Splenic DC were cocultured with apoptotic EG7 (apoEG7) cells at a 1:1 ratio in the presence or absence of IFN-I (5 × 10^5 U/ml) for 3–18 h at 37°C, 5% CO2. To assess uptake in vitro, DC were first separated from excess of uneaten apoptotic fragments by Nycodenz density-gradient centrifugation. IFN-I (10 U/ml) was added to the mixture of antibody, biotin anti-I-A, streptavidin–peroxidase, and biotin anti-I-A, to detect the presence of apoptotic bodies (CFSE+) in CD11c+CD8+ cells; data not shown). In some experiments, CD11c+ cells were purified by this method are virtually free from T cell contaminants (95–98% CD11c+ cells). The DC purified by this method are virtually free from T cell contaminants (<0.1% CD3+ cells; data not shown). In some experiments, CD11c+ cells were further sorted into CD8a+ and CD8α− subsets using FACS Aria cell sorter (BD Biosciences), yielding 99% purity. CD8 T cells from OT-I splenocytes and lymph node (LN) cells were purified using anti-CD8 microbeads (Miltenyi Biotec). Purity routinely ranged: 95–98%.

OT-I T cell priming

For in vitro priming, OT-I CD8 T cells were labeled with CFSE (1 μM) and then seeded in 96-well U-bottomed plates (10^5 cells/well) together with decreasing numbers of DC previously loaded with apoEG7 cells. Cocultures were performed in triplicate and incubated at 37°C, 5% CO2 for 3 d. Cells were then harvested and stained with anti-CD8, CD25, and CD69 mAbs and analyzed by FACS. CFSE dilution and phenotypic activation in CD8 T cells were determined by FACS at 3 h later, mice were sacrificed and splenic DC were analyzed by FACS for each experimental point. For quantization, threshold cycle (Ct) values were determined by the Sequence Detection System software (Applied Biosystems), and ΔCt was obtained by subtracting Ct of reference gene, β-actin, from Ct of target gene. Gene expression was represented as relative amount of mRNA normalized to β-actin and was calculated as 2−ΔΔCt (16).

Results

Apoptotic EG7 cells are selectively captured by CD8a+ DC in vitro and in vivo

To evaluate the effects of IFN-I on cross-presentation of Ag derived from apoptotic cells, we used OVA-expressing EG7 thymoma cells induced to apoptosis by UV irradiation. In mice, CD8a+ DC are the specialized population for cross-presentation, although such feature may not be attributed to an exclusive ability of these cells to capture exogenous Ag (17). However, accumulating evidence suggests that apoptotic cells are captured preferentially by CD8a+ DC (18, 19). We initially evaluated which DC subsets were able to capture apoEG7 cells. To this end, we cocultured magnetically sorted CD11c+ splenic DC with apoEG7 cells for 3 h, then removed the excess of uneaten apoptotic fragments by Nycodenz density-gradient centrifugation, to enrich the DC fraction (80%; Fig. 1A), and analyzed the uptake in the two DC subsets by FACS. As expected, CD8a+ cells, but not CD8α− cells, efficiently captured apoEG7 cells in vitro (Fig. 1A) and in vivo (Fig. 1B). To confirm the exclusive ability of CD8a+ DC to phagocytose apoEG7 cells, we repeated the experiment with DC from IRF-8–deficient (IRF-8−/−) mice, indicating that apoEG7 cells could be captured exclusively by CD8a+ DC (18, 19). We next evaluated the effects of IFN-I on phagocytosis of apoEG7 cells by CD8a+ DC. To this end, we cocultured magnetically sorted CD11c+ splenic DC with apoEG7 cells for 3 h, then removed the excess of uneaten apoptotic fragments by Nycodenz density-gradient centrifugation, to enrich the DC fraction (80%; Fig. 1A), and analyzed the uptake in the two DC subsets by FACS. As expected, CD8a+ cells, but not CD8α− cells, efficiently captured apoEG7 cells in vitro (Fig. 1A) and in vivo (Fig. 1B). To confirm the exclusive ability of CD8a+ DC to phagocytose apoEG7 cells, we repeated the experiment with DC from IRF-8–deficient (IRF-8−/−) mice, indicating that apoEG7 cells could be captured exclusively by CD8a+ DC (Fig. 1C).

IFN-I promote Ag retention by CD8a+ DC after uptake of apoEG7 cells

Next, we evaluated the effects of IFN-I on phagocytosis of apoEG7 by CD8a+ DC. To this end, we cocultured CFSE-labeled apoEG7 with splenic DC in the presence or absence of IFN-I for 3–18 h and measured the internalization of apoptotic bodies by DC. We found that although IFN-I treatment did not significantly affect the uptake of CFSE+ apoEG7 cells by CD8a+ DC, as revealed by FACS at 3 h, it determined significant retention of phagocytosed material after 18 h (Fig. 2A). In fact, whereas at 3 h the percentages of CD8αCFSE− were comparable in both cultures, at 18-h
coculture 20.2% CD8α+CFSE+ were found in CD8α+ DC cultured in the presence of IFN-I with respect to only 8.3% CD8α+CFSE+ in control cells (Fig. 2A). Hence, in IFN-treated samples, the percentage of CFSE+CD8α+ cells found at 18 h was similar to that at 3 h (20.2 versus 21%; Fig. 2A), whereas in untreated controls the amount of CFSE+CD8α+ cells dropped considerably at 18 h with respect to 3 h (8.3 versus 20.1; Fig. 2A). Analysis of mean fluorescence intensity (MFI) in CFSE+CD8α+ cells revealed that IFN-treated DC exhibited increased levels of green fluorescence with respect to untreated cells, suggesting a larger number of antigenic material carried (Fig. 2B). CLSM observations further evidenced a higher frequency of CFSE+ particles within the intracellular compartment of IFN-treated DC, with respect to untreated cells at 18 h (Fig. 2C). Densitometric analysis of CFSE fluorescence revealed a significant increase in the green fluorescence intensity retrieved in phagocytic IFN-treated DC, with respect to untreated DC (NT-DC), indicating a larger quantity of antigenic particles per cell at 18 h (Fig. 2D). This finding led us to hypothesize that IFN-I may control the persistence of apoptotic bodies within the phagosomal compartments of DC. Alternatively, although unlikely, IFN-I may prolong the endocytic activity of DC throughout the 18-h culture, meaning that the DC were continuously eating and processing the Ag. To test this, we cocultured DC with CFSE+ apoEG7 cells for 3 h, with or without IFN-I, then removed the excess of “uneaten” apoptotic bodies from the culture by density-gradient centrifugation and left the DC alone in the identical culture medium (containing or not IFN-I) for the remaining 15 h of culture (3 h + 15 h). In this setting, no more apoptotic bodies were available for DC to eat, thus meaning that the antigenic material to be retrieved within DC at 18 h would be the result of the unprocessed Ag only. Remarkably, the percentages of CD8α+ CFSE+ retrieved in samples of IFN-treated DC in which apoEG7 cells had been withdrawn at 3 h (3 h + 15 h) were similar to those found with DC undergoing continuous 18-h coculture (18 h), indicating that IFN-I effectively acted by prolonging Ag retention in CD8α+ DC (Fig. 2E). In contrast, untreated CD8α+ DC lost ~70% of antigenic cargo either in the continuous 18 h or in the 3 h + 15-h coculture setting with respect to 3-h cocultures (Fig. 2E).

To assess whether IFN-I could prolong Ag persistence within phagocytic CD8α+ DC in vivo, we injected CFSE-labeled apoEG7 cells in combination or not with IFN-I and analyzed the uptake by splenic CD8α+ DC after 3 and 18 h. Similarly to what was observed in vitro, we found that the uptake of apoptotic cells by CD8α+ DC in vivo was only marginally increased by IFN-I, as revealed by the percentage of phagocytosis found in IFN-I–treated and untreated DC at 3 h postinjection (0.34 versus 0.55%; Fig. 2F). Remarkably, CD8α+ DC from mice exposed to IFN-I treatment almost completely retained the antigenic cargo after 18 h (0.47%; Fig. 2F), whereas this was completely lost by cells of mice injected with apoEG7 alone (0.06%; Fig. 2F).

**Role of intracellular pH alkalinization in IFN-induced prolonged Ag persistence**

CD8α+ DC are thought to possess specialized machinery to direct endocytosed Ag into the MHC class I presentation pathway. In this regard, the Ag cross-presentation pathway is thought to be critically dependent on low proteolytic activity of lysosomal enzymes, a process requiring a high phagosomal pH. This physiological condition results in enhanced Ag storage within the intracellular compartments, allowing DC to display peptide within both MHC-I and MHC-II complexes (7). To address whether IFN-I treatment could modulate intracellular pH in phagocytic DC, at various times of coculture with apoEG7 cells we treated DC with LysoSensor green, a fluorescent acidotropic probe exhibiting a pH-dependent increase in MFI upon acidification. As shown in Fig. 3A, at 5–7 h postcoculture, DC treated with IFN-I exhibited a significant drop in the MFI, implying a more alkaline phagosomal pH with respect to untreated cells. To further assess the role of intraphagosomal pH in IFN-induced Ag retention by CD8α+ DC, we used DPI, an inhibitor of the activity of NADPH oxidase 2 (NOX2), a flavin-containing enzyme known to control pH alkalinization in CD8α+ DC phagosomes (21). DPI was added to IFN-treated and untreated DC-apoEG7 cocultures at 3 h, in order not to interfere with phagocytosis, and left until 18 h. As shown in Fig. 3B, addition of DPI significantly reduced the percentages of CFSE+CD8α+, but not of CFSE+CD8α−, cells in IFN-treated cultures, indicating a decrease

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**FIGURE 1.** Selective uptake of apoEG7 cells by CD8α+ DC. A, Purified splenic CD11c+ DC were cocultured at a 1:4 ratio with CFSE-labeled apoEG7 cells for 3 h at 37˚C or at 4˚C, then separated from apoptotic cells by Nycodenz centrifugation, surface stained for CD8 and CD11c expression, and analyzed by FACS for uptake by gating on CD11c+ cells. Plots represent the percentage of CD11c+ DC engulfing apoEG7 cells (CFSE+). One representative experiment of nine is shown. B, Naive C57BL/6 mice were injected i.v. with 5 × 10⁶ CFSE-labeled apoEG7 cells or PBS. Three hours later, splenic DC were magnetically sorted and uptake was evaluated by FACS analysis on gated CD11c+ cells. Representative data from four experiments are shown. C, Uptake by splenic CD11c+ DC from IRF-8−/− or WT C57BL/6 mice after 3-h coculture with CFSE-labeled apoEG7 cells at 37˚C. One representative experiment of three is shown.
in Ag retention. As expected, DPI also decreased the proportion of CFSE+CD8α+ DC in cultures not containing IFN-I (Fig. 3B). These data strongly suggest that the effects of IFN-I on Ag retention by CD8α+ DC can involve a regulatory mechanism of pH within phagosomal vesicles.

**IFN-I sustain the survival of Ag-bearing CD8α+ DC**

In addition to the effects on Ag retention, we found that IFN-I treatment resulted in increased numbers of total CD8α+ DC recovered after 18-h coculture with apoEG7 cells, as revealed by percentages in IFN-I–containing cultures with respect to untreated cultures (26.7 versus 15.3%; Fig. 4A), and by absolute numbers of CD8α+ DC retrieved in the cultures (Fig. 4B). These observations suggest that IFN-I could also promote the survival of Ag-bearing CD8α+ DC, namely DC that have engulfed apoEG7 cells. To test this, we analyzed the mortality of sorted CD8α+ and CD8α+2 DC after culture with CFSE+ apoEG7 cells in the presence or absence of IFN-I. As revealed by PI staining, addition of IFN-I significantly decreased the percentage of dying CD8α+ DC (32.5%), with respect to untreated cells (50.3%; Fig. 4C). Conversely, IFN-I
caused increased mortality levels of CD8α2 DC (61.5%), with respect to untreated controls (37.5%; Fig. 4C), in keeping with the reduced numbers of CD8α2 DC retrieved in the cultures containing IFN-I (Fig. 4B). Of note, among CD8α+ DC, IFN-I selectively promoted the survival of phagocytic cells, as revealed by reduced PI staining in CD8α+CFSE+ cells (27.8%) with respect to untreated controls (60.2%), but not that of nonphagocytic cells, as revealed by similar percentages of PI+ cells in IFN-treated versus untreated CD8α+CFSE− (41.1 versus 47.1%; Fig. 4D). Because we previously reported that IFN-I promote apoptosis of bystander DC, such as DC not bearing Ag, by downmodulating antiapoptotic molecules of the bcl-2 family (15), we analyzed whether IFN-I could modulate the expression of these genes in Ag-bearing CD8α+ DC. As illustrated in Fig. 4E, IFN-I treatment markedly increased the expression of bcl-2 and bcl-xL in CD8α+ DC after 3- and 18-h culture. In contrast, IFN-I treatment decreased both bcl-2 and bcl-xL in CD8α− DC, to be considered as bystander DC in this setting (Fig. 4E). These results confirm our previous findings on the proapoptotic effects of IFN-I on bystander DC (15) and suggest that these cytokines may instead act as a survival factor for Ag-bearing DC.

IFN-I promote cross-presentation of cell-associated OVA by CD8α+ DC and stimulate CD8 T cell priming

Previous studies have shown that the expression of SIINFEKL peptide of OVA in association with MHC-I (MHC-OV Ap) on DC membrane can be highly increased by LPS treatment, suggesting that signals capable of activating DC could also enhance cross-presentation (22). Thus, to assess whether IFN-I affected cross-presentation of apoEG7-derived antigenic material, we analyzed MHC-OV Ap surface expression on CD8α+ DC by FACS. Remarkably, IFN-I strongly enhanced the levels of MHC-OV Ap in CFSE+CD8α+ DC, with respect to untreated cells (49.7 versus 8.7% expressing cells; Fig. 5A). Of note, IFN-I–induced upregulation of MHC-OV Ap expression on CD8α+ DC was selectively abolished by blocking with Ab to mouse IFN-I, demonstrating that this effect was specifically mediated through IFN-I receptor (Fig. 5B).
Moreover, expression of MHC-OVAa in IFN-treated phagocytic CD8a+ DC was also inhibited by DPI, supporting the involvement of intraphagosomal pH regulation by IFN-I in stimulating cross-presentation (Fig. 5A). To become competent for cross-priming, DC require a license signal inducing full maturation. In this regard, IFN-I have been widely described to activate DC for induction of Ag-specific T and B cell immunity (23, 24). Thus, we analyzed whether IFN-I exposure resulted also in activation of CD8a+ DC that had taken up apoEG7 cells and found noticeable activation of these cells, as revealed by increased expression of CD40 and CD86 molecules with respect to untreated cells (Fig. 5B). Of note, IFN-I induced far more marked phenotypic activation in phagocytic CD8a+ DC, with respect to non-phagocytic CD8a+ DC in vitro (Fig. 5B) and in vivo, when injected in combination with apoEG7 cells (Fig. 5C).

To investigate the ability of IFN-treated CD8a+ DC to cross-prime CD8 T cells against cell-associated OVA, we cultured DC loaded with apoEG7 in the presence or absence of IFN-I with CFSE-labeled OT-I lymphocytes and analyzed OVA-specific responses. Consistent with the increased levels of cross-presented OVA, IFN-treated DC stimulated higher proliferation of OT-I cells, as compared with untreated controls, again indicating increased OVA cross-presentation (Fig. 6A). Of interest, OT-I CD8 T cells responding to IFN-DC, unlike those responding to NT-DC, exhibited an activated phenotype, as revealed by expression of CD25 and CD69 (Fig. 6A). Cross-priming of OT-I cells in response to IFN-treated DC was also confirmed by IFN-γ ELISPOT assay (Fig. 6B). Remarkably, even IFN-DC to whom apoEG7 cells were withdrawn after 3 h coculture (IFN-DC 3 h + 15 h; Fig. 6B) were potent stimulators of IFN-γ-specific effector response, in accordance with their mature phenotype (Supplemental Fig. 1), priming OT-I cells as efficiently as IFN-treated DC from the continuous coculture (IFN-DC 18 h; Fig. 6B). In contrast, apoEG7-DC alone induced poor OT-I effector response (NT-DC 18 h; Fig. 6B). Next, we examined the capacity of IFN-treated DC to induce CD8 T cell cross-priming in vivo when injected into naïve C57BL/6 mice adoptively transferred with CFSE-labeled OT-I cells. In an attempt to use as stimulators apoEG7-loaded DC generated by 18-h coculture with or without IFN, we failed to observe measurable proliferative responses in vivo (data not shown). Thus, we immunized mice by injecting apoEG7-DC (i.e., DC loaded with apoEG7 cells by 3-h coculture) alone or combined with IFN-I, so as to prolong cytokine exposure in vivo. Three days later, we measured OVA-specific T cell priming in draining or, as a control, distal LN by FACS. Remarkably, only mice immunized with apoEG7-DC plus IFN-I displayed sustained proliferative response in draining LN (Fig. 7A), resulting also in activation of OT-I cells, as evidenced by CD25 upregulation in proliferating cells (11.5%; Fig. 7B). In contrast, mice injected with apoEG7-DC alone failed to induce OT-I proliferation in vivo, confirming the tolerogenic potential of these cells (Fig. 7A). As expected, no significant OT-I cell response could be detected in distal LN from mice immunized with apoEG7-DC plus IFN-I (Fig. 7A, 7B). Lastly, we examined the ability of IFN-I to license DC for cross-priming in vivo by injecting apoEG7 cells, alone or combined with IFN-I, in wild-type (WT) C57BL/6 or IRF-8-/- mice, whose DC are unable to capture apoEG7 cells. Strikingly, IFN-stimulated OT-I cross-priming was detected in WT, but not in IRF-8-/- recipients, as revealed by substantial proliferation of adoptively transferred OT-I lymphocytes in mice injected with apoEG7 plus IFN-I (70%; Fig. 7C), indicating that the cytokines were mediating this effect through CD8a+ DC stimulation.

**Discussion**

Cells dying purposefully by apoptosis are thought to be phagocytosed by mechanisms that fail to incite inflammatory or immune reactions. Hence, clearance of apoptotic cells by phagocytes results in anti-inflammatory and immunosuppressive effects, thus hampering the onset of T cell effector responses. This occurs because engulfment of apoptotic material results in lack of induction of proinflammatory cytokines or even in the release of immunoregulatory factors that maintain DC in an immature state (25). The findings reported in this study demonstrate that IFN-I can act as a powerful switch signal for DC promoting cross-priming in vivo against a largely tolerogenic type of Ag, such as Ag derived from tumor apoptotic cells. In doing so, IFN-I control CD8a+ DC activity at three distinct levels. First, IFN-I treatment prolongs the intracellular persistence of antigenic particles engulfed by phagocytic CD8a+ DC, as revealed by increased levels of CFSE fluorescence intensity in phagocytic CD8a+ DC after 18-h culture. As a result, IFN-treated DC exhibited enhanced cross-presentation of apoptotic cell-derived OVA, as evidenced by surface expression of MHC-I–OVAa complexes and by induced OT-I cell proliferation. Ag persistence is a crucial event regulating the magnitude of cross-presentation and is promoted by a reduced lysosomal proteolysis that delays the degradation of phagocytosed Ag, in a process requiring a limited phagosomal acidification. As a mechanism regulating intraphagosomal pH, the NOX2 enzyme was shown to induce active alkalization of the phagolysosomal compartments selectively in DC (7). In this study, Ag persistence induced by IFN-I strongly correlated with pH alkalization and was restrained by addition of the NOX2 inhibitor DPI, resulting in
reduced cross-presentation of EG7-derived OVA by CD8α+ DC. Previous studies reported that signaling through TLR2, TLR3, TLR4, and TLR9 enhances Ag uptake, resulting in more efficient cross-presentation (26). In the current study, we show that IFN-I enhance CD8α+ DC cross-presentation of tumor apoptotic cell-derived Ag mainly affecting Ag processing. Consistent with this view, studies on human DC indicate that IFN-I can affect the expression of a number of genes associated with processing as well as the expression of inducible proteasome subunits (11, 27, 28). It is worth mentioning that in our setting, withdrawal of apoEG7 cells from the coculture at 3 h did not prevent IFN-induced Ag retention and OVA cross-presentation in CD8α+ DC, provided that IFN-I were maintained in the culture for the remaining 15 h. In fact, removal of both apoEG7 cells and IFN-I after the 3-h culture resulted in only partial Ag retention and no DC activation and cross-presented OVA (Supplemental Fig. 2). This observation suggests that IFN-I exposure may be required all through the Ag-processing phase to lead to MHC-I cross-presentation and DC activation.

As a second effect, IFN-I promoted the survival of phagocytic, but not of nonphagocytic, CD8α+ DC, through the selective modulation of the apoptosis-related genes Bcl-2 and Bcl-xL. This finding, as opposed to our previous finding showing IFN-I exerting proapoptotic effects on bystander (i.e., in the absence of Ag) DC, suggests an elegant regulatory mechanism by which IFN-I selectively sustain the life span of Ag-bearing DC for induction of effective immune responses, while favoring a rapid clearance of steady-state DC (15). The duration of DC life span critically regulates the efficiency of cross-priming and the outcome of adaptive immunity, although little is known about the role of Ag persistence in this process (29). A recent study in mice infected with bacillus Calmette-Guérin has shown that Ag persistence in infected DC is strictly correlated with DC survival (30). Thus, these data suggest that duration of Ag persistence and survival of DC may be two linked processes regulating the extent of Ag presentation and cross-presentation. In this respect, the effects of IFN-I in promoting both Ag persistence and survival of CD8α+ DC may be regarded as two tightly correlated events, because in our studies increased Ag retention by the CD8α+ DC strongly correlated with a longer life span of these cells, although further investigations are required to determine which process is causative of the other.

A third process accounting for IFN-I effects is the activation of DC, revealed by upregulation of both costimulatory markers and proinflammatory cytokines (Supplemental Fig. 3), that provide a license signal for DC to cross-priming, consistent with previous reports showing IFN-I to be a powerful stimulus for DC activation (9, 24). Multiple events have been described licensing DC for cross-priming that include CD40L engagement by CD4 Th cells,
8 stimulation by NK cells, TLR triggering, and exposure to soluble factors released upon injury or infection (5, 31). Among these soluble mediators, IFN-I have been described to be particularly efficient in inducing cross-priming in a CD4 T cell-independent manner, implying a faster immune reaction (9, 10). Besides the appreciated effects in promoting cross-priming against soluble or viral Ag, some recent evidence suggests that IFN-I may also affect cross-presentation of cell-associated Ag (11). Of note, a recent study on the newly described mouse merocytic DC subset has shown that these cells are endowed with potent ability to prime both CD4 and CD8 T cells against tumor cell-associated Ag partly through their ability to produce IFN-I upon engulfment of apoptotic tumor cells (32). Moreover, cellular association of dsRNA with irradiated EG7 cells was shown to elicit CD8 T cell responses in vivo that were dependent on dsRNA-induced IFN-I secretion by DC (33). Finally, we have recently reported that IFN-I can greatly enhance cross-presentation and CD8 T cell cross-priming by stimulating CD8α+ DC that have engulfed tumor cells undergoing an immunogenic type of apoptosis by chemotherapy treatment (13). The present study extends this view to demonstrate that IFN-I can act as a switch signal for CD8α+ DC cross-presenting tumor apoptotic cell-derived Ag converting the response into cross-priming.

The property of innate stimuli, such as those triggering TLR3, TLR4, and TLR9, to stimulate T cell responses has been shown to be largely dependent on the induction of endogenous IFN-I (34–36). Of interest, TLR agonists have been shown to stimulate cross-priming either at the level of DC licensing or by enhancing Ag cross-presentation (37, 38). Our results suggest that IFN-I induce cross-priming stimulating both processes in CD8α+ DC and, additionally, by sustaining the life span of Ag-bearing DC. Because mouse CD8α+ DC are specialized for MHC class I presentation and CD8 T cell activation, these cells are regarded as the ideal DC subtype for targeted vaccination to generate effector CTL responses. Given the recent discovery of human equivalents of mouse CD8α+ DC (39–42), our studies provide new knowledge on IFN-I properties to be exploited for the design of innovative clinical protocols in which the generation of effective cytotoxic immunity is critically required, such as in anticancer treatments. Importantly, a role for IFN-I in induction of autoimmunity has recently emerged. In fact, therapeutic treatment with IFN-I, especially IFN-α, in cancer and other pathologies has been associated with the onset of collateral autoimmune disorders, leading to the hypothesis that these effects may be due to a hyperstimulation of immune cells, such as DC, by these cytokines (43). The results reported in this study support this concept and provide a potential mechanism by which IFN-I may induce autoimmune reactions, namely through the enhancement of DC activation and the presentation of self Ag derived from cells undergoing constitutive apoptosis.

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