Maturation Determines Their Longevity

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Exposure of Myeloid Dendritic Cells to Exogenous or Endogenous IL-10 during Maturation Determines Their Longevity

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Abstract

Dendritic cells (DCs) are essential for the initiation of primary adaptive immune responses, and their functionality is strongly down-modulated by IL-10. Both innate and adaptive immune signals trigger the up-regulation of antiapoptotic Bcl-2 family members to facilitate the survival of DCs after maturation. However, whether IL-10 alters the expression of apoptotic-related genes in maturing DCs has not been determined. In this study, we demonstrate that spontaneous apoptosis rapidly occurred in myeloid DCs exposed to exogenous IL-10 upon maturation. Microarray analysis indicates that IL-10 suppressed the induction of three antiapoptotic genes, bcl-2, bcl-xL, and bfl-1, which was coincident with the increased sensitivity of mature DCs to spontaneous apoptosis. IL-10 markedly inhibited the accumulation of steady state Bcl-2 message and protein in myeloid DCs activated through TLRs or TNFR family members, whereas exogenous IL-10 affected Bcl-xL expression in a moderate manner. In contrast, bcl-2 expression of plasmacytoid DCs was less sensitive to the effects of IL-10. We further show that autocrine IL-10 significantly limited the longevity of myeloid DCs and altered the expression kinetics of Bcl-2 but not Bcl-xL in maturing DCs. We conclude that the degree of IL-10 exposure and/or the level of endogenous IL-10 production upon myeloid DC maturation play a critical role in determining DC longevity. This regulatory mechanism of IL-10 is associated with the dynamic control of antiapoptotic Bcl-2 proteins.

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Dendritic cells (DCs) constitute a highly specialized cell population that plays a key role in determining the quality and magnitude of immune reactions against foreign or self Ags (1, 2). The immunogenicity (or tolerogenicity) of DCs is highly associated with their cytokine and chemokine production, costimulatory molecule expression, and longevity. This functional plasticity largely depends on the ability of DCs to respond to various extracellular cues during the discrete stages of their developmental maturation. Although each DC subtype, including myeloid DCs, plasmacytoid DCs (PDCs), and Langerhans DCs, possesses certain unique functionalities, they commonly share the capacity to process and present Ags to naive T cells (2). The encounter of Ag-bearing DCs with circulating Ag-specific T cells in the draining lymph nodes is a rare event. The lifespan of mature DCs is also quite limited with estimates of 3 days in vivo (3, 4). The lifespan of DCs, shaped by the signals they receive from the microenvironment, therefore influences the scale and sustainability of DC-induced immune reactions (5–7).

Apoptosis is critical for maintaining the homeostasis of the immune system. Directing the lifespan of DCs by means of apoptosis serves as an efficient tool to control the initiation and termination of immune and inflammatory reactions. The lifespan of the mature DCs can be prolonged when cells receive survival signals from T cells, including CD40L and TNF-related activation-induced cytokine, through the up-regulation of anti-apoptotic Bcl-xL (7, 8). DC turnover is controlled by two distinct pathways: a Bcl-xL-dependent survival pathway triggered by signals through TLRs or CD40; and a Bcl-2-dependent molecular timer that is only seen in TLR ligand-activated DCs (9). The extracellular signals that dynamically modulate these molecules in DCs are unknown.

IL-10 is an anti-inflammatory cytokine that potently modulates the expression of cytokines, chemokines, and surface molecules of macrophages and DCs. IL-10 exposure reduces the capacity of DCs to activate and sustain immune and inflammatory responses (10). IL-10 is an important immune modulator that strikes a balance between immune protection and immunopathology. Conversely, failure to generate protective immunity in certain disease states has been linked to interference by IL-10. Elevated levels of IL-10 are found in many cancer patients (11). Many intracellular pathogens have co-opted IL-10 to facilitate their infection or long-term persistence. Respiratory syncytial virus, murine CMV, Mycobacterium tuberculosis, and Listeria monocytogenes infect macrophages and enhance IL-10 production by these cells (12–15). Some DNA viruses, including EBV and human CMV, encode viral IL-10 homologs to engage the cellular IL-10R (16, 17). Although human CMV-encoded IL-10 (cmvIL-10) shares only 27% amino acid sequence with its cellular counterpart (16, 18), its homodimer engages the ligand-binding subunit of IL-10R with as high affinity as human IL-10 (hiIL-10; Ref. 19). hiIL-10 and cmvIL-10 show no...
differences in their ability to suppress phenotype maturation and proinflammatory cytokine production of DCs (20, 21). However, whether cmvIL-10 encodes additional modulatory functions besides those of hIL-10 remains unknown.

Promoting apoptosis of mature DCs may be one mechanism of IL-10 regulation that can appropriately benefit the maintenance of immune homeostasis, or that inappropriately alters immune reactions during diseases or microbial infection. Compromised longevity of mature DCs, even at a moderate level, could strongly down-grade the scale of DC-induced adaptive immunity. Previous reports have proposed that IL-10 can induce apoptosis of myeloid DCs (20), Langerhans DCs (22), and PDCs (23–25). In this study, we demonstrate that both hIL-10 and cmvIL-10 trigger apoptosis of mature myeloid DCs. The mechanism of IL-10-induced cell loss is likely mediated through the suppression of bcl-2, bcl-xL, and bfl-1 induction associated with DC maturation. Further, we show that endogenous IL-10, which is strongly induced by engagement of TLRs on myeloid DCs, controls the lifespan of mature DCs in an autocrine fashion. Interestingly, endogenous IL-10 alters the up-regulation of Bcl-2, but not Bcl-xL. These data suggest that the scale of IL-10 exposure throughout the maturation process determines the lifespan of mature DCs, likely through the dynamic modulation of Bcl-2 expression.

Materials and Methods
Monocyte-derived DC cultures and blood DC isolation
Leukocyte-enriched buffy coats from healthy individuals were obtained from the Stanford Blood Center (Mountain View, CA). PBMC preparation, CD14+ monocyte isolation, and the generation of monocyte-derived DC (MDDC) cultures were performed as described previously (21). Primary DC populations were positively isolated using the Blood dendritic Cell Isolation Kit II (Miltenyi Biotec). Labeled cells were purified with an autoMACS separator using the programs recommended by the manufacturer. PDCs and CD11c+ myeloid DCs were positively isolated from PBMCs using the BDCA-4 and BDCA-1 Cell Isolation kits, respectively (Miltenyi Biotec). Yields of PDCs and CD11c+ DCs were 98% and 98%, respectively.

DC activation and treatment
For myeloid DC activation, 10 ng/ml LPS from Escherichia coli O117:B8, 5 μg/ml lipoteichoic acid (LTA) from Bacillus subtilis, 50 μg/ml polymyxin-polyethylic acid (poly(LC), all purchased from Sigma-Aldrich), 1 μg/ml purified recombinant human soluble CD40L (sCD40L; Research Diagnositc), 100 μg/ml lipoteichoic acid (LTA) from Escherichia coli, 20 μg/ml poly(I:C); all purchased from Sigma-Aldrich), 50 ng/ml recombinant hIL-10 or cmvIL-10 proteins were added to treatment, 5 ng/ml recombinant hIL-10 or cmvIL-10 (R&D Systems) were added. A-class CpG oligodeoxynucleotides (50 μg/ml; R837; Coley Pharmaceutical Group), imiquimod (5 μg/ml; InvivoGen), or heat-inactivated influenza virus (A/Mem/71) equivalent to 1000 U/ml purified recombinant human TNF-α (R&D Systems) were added. Biotin-labeled cRNA was purified and fragmented, and hybridized to Human Genome Focus Arrays (Affymetrix) according to the manufacturer’s protocol. The arrays were washed and stained on a Fluidics Station 450 and scanned on a GeneChip Scanner 3000 (Affymetrix). The Focus Array represents ~8400 well-characterized genes. Data analysis of gene expression arrays was conducted using ArrayAssist software 3.3 (Stratagene). Probe level analysis, data normalization, and probe set summarization were conducted using GC-RMA. Raw signal values for this entire data set are publicly available (NCBI GEO accession number GSE7095).

Quantitative real-time PCR assay
Steady state bcl-2 and bcl-x mRNA levels were measured by quantitative real-time PCR assays in a Sequence Detection System (ABI/Prism 7900HT; Applied Biosystems). Primers and probes were designed with Primer Express software (Applied Biosystems). Primer and probe sequences specific to both α and β isoforms of bcl-2 transcript variants are: forward 5′-CGGCCCTTGAGCTGACTGTA-3′; reverse 5′-CCCCAGCC TCCGTTTAATCCTG-3′; probe 5′-tetrachloro-6-carboxylfluorescein-CTGA ACGCTTCTGACG-3′; forward 5′-tetrachloro-6-carboxylfluorescein-AGA GCCAAGGCGCAACG-3′; Primer and probe sequences specific to both long (bcl-xL) and short (bcl-xS) isoforms of bcl-x mRNA are: forward 5′-ATGGGACAATGCGACGACG-3′; reverse 5′-TCCAGG ACCACGGTTTGA-3′; probe 5′-tetrachloro-6-carboxylfluorescein-AGA GCCAAGGCGCAACG-3′. Relative copy number of mRNA of each sample was calculated according to the standard curves generated from 10-fold serial dilutions (from 10⁶ to 1 copies/μl) of plasmids containing bcl-2 (pWCC254) or bcl-x (pWCC255) amplicons.

RNA isolation and processing
Total RNA was extracted from DCs at various time points after stimulation using the RNeasy Mini kit (Qiagen). RNA quality and integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies). Only high-quality RNA, having a 28S/18S RNA ratio ≥1.8 and an OD260/OD280 ratio >2.0, was utilized. For microarray analysis, purified total RNA (10 μg) was converted to cDNA and to biotin-labeled cRNA with a BioArray HighYield RNA Transcript Labeling kit (Enzo Biochem). For quantitative real-time PCR analyses, RNA treatment and cDNA synthesis were performed as described previously (21).

DNA microarray analysis
Microarray analysis was performed with samples from two independent experiments. Biotin-labeled cRNA was purified, fragmented, and hybridized to Human Genome Focus Arrays (Affymetrix) according to the manufacturer’s protocol. The arrays were washed and stained on a Fluidics Station 450 and scanned on a GeneChip Scanner 3000 (Affymetrix). The Focus Array represents ~8400 well-characterized genes. DNA analysis of gene expression arrays was conducted using ArrayAssist software 3.3 (Stratagene). Probe level analysis, data normalization, and probe set summarization were conducted using GC-RMA. Raw signal values for this entire data set are publicly available (NCBI GEO accession number GSE7095).

Flow cytometry
Four-color flow cytometry was performed using a FACSCalibur cell sorter operated by CellQuest software (BD Biosciences) using directly conjugated mAbs against CD3, CD4, CD11c, CD14, CD83, CD86, CD123, Bcl-2, active caspase-3, and HLA-DR (BD Biosciences). For detection of apoptosis, cells were stained with FITC-conjugated annexin V and propidium iodide (PI) using the Apoptosis Detection Kit (BD Biosciences). Intracellular staining for Bcl-2 and active caspase-3 was performed with the Fixation/Permeabilization Solution kit (BD Biosciences) according to the manufacturer’s instructions after surface staining with the appropriate mAbs. Appropriate isotype-matched mAbs were used as controls for all immunostainings. Data were analyzed and illustrated using FlowJo software (Tree Star).
Immunoblotting

DC lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Nonspecific binding was blocked by overnight incubation of membranes at room temperature with 5% skim milk and 0.1% Tween 20 in PBS, pH 7.4. The following Abs were used for immunoblotting: anti-Bax (clone 3), anti-Bid (clone 7), anti-Bcl-x, anti-Grh2 (clone 81) (BD Biosciences); anti-Bcl-2 (clone 100; Upstate). Immunodetection was performed using the appropriate peroxidase-conjugated secondary Ab and the Visualizer Western Blot Detection Kit (Upstate). Chemiluminescence of blotted membranes was scanned with a Typhoon 9410 variable mode imager (GE Healthcare). Protein amounts were quantified by determining the intensity of bands within their linear detection range (ImageQuant software; GE Healthcare).

ELISA

The levels endogenous IL-10 secreted by DCs were quantified using ELISA kits purchased from U-CyTech Biosciences. The limits of detection of the ELISA assays were 5 pg/ml.

Statistical analysis

Statistical comparisons between two groups were performed by the Student t test (paired, two-tailed). One-way ANOVA followed by Tukey’s multiple comparison tests or by Dunnett’s multiple comparison tests were used for statistical analyses between groups greater than two, as appropriate. In the Dunnett posttests, data from samples treated with stimuli and isotype control mAb were chosen as controls. All statistic analyses were performed using Prism Software (GraphPad).

Results

IL-10 induces apoptosis of activated DCs

The first series of experiments examined the proapoptotic effects of exogenous IL-10 on LPS-activated MDDCs. To compare the effects of host vs viral IL-10, MDDC cultures were concomitantly treated with LPS and either hIL-10 or cmvIL-10 for 12 and 24 h and assessed by FACS for apoptosis. Annexin V and PI staining and intracellular active caspase-3 staining indicated that DCs exposed to hIL-10 or cmvIL-10 upon LPS activation were prone to apoptosis. As shown in Fig. 1A, after 12 h of LPS activation, MDDC cultures treated with IL-10 contained higher frequencies of annexin V-positive cells than cells stimulated with LPS alone. The difference in the frequencies of cells undergoing apoptosis was more apparent when cells were activated for 24 h (Fig. 1A). In contrast, IL-10 treatment did not promote the spontaneous apoptosis of unstimulated immature DCs (data not shown).

We also looked at the activation of caspase-3, a protease that is activated by cleavage during the early stages of apoptosis, to confirm the occurrence of apoptosis induced by IL-10. Cells were surface stained with HLA-DR and CD86 and followed by intracellular staining with mAb specific for activated caspase-3. The results of intracellular active caspase-3 staining correlated with the annexin V staining data. After 12 h of LPS activation and IL-10 treatment, a low level of activated caspase-3-positive cell became detectable by FACS. At 24 h, marked increases in the frequencies of DCs with activated caspase-3 were observed in the IL-10-treated groups (Fig. 1B). Viable cell counts were conducted to confirm the results of apoptotic analysis. Cell recovery data showed that exogenous hIL-10 treatment significantly reduced the viable cell numbers in LPS-activated MDDC culture by an average of 47% (range, 43–54%), relative to the untreated culture (Fig. 2A). The maturation process converts DCs to a physiological status that is distinctly different from that of immature DCs. Mature DC turnover appeared to be more rapid, in contrast to the turnover rate of immature DCs. A measurable cell loss was observed when immature DCs received activation signals through TLR4, regardless of exogenous IL-10 treatment. DC activated by LPS for 48 h resulted in an average of 21% cell loss (range 17–26%; Fig. 2A). Even though a higher frequency of annexin V-positive cells was observed in the untreated group than the LPS-treated group (Fig. 1A), the viable cell numbers of untreated cultures remained stable for up to 48 h (Fig. 2). At 72 h after onset, a marked decline of the cell number was observed in the untreated group (Fig. 2B), which may be attributed to the GM-CSF and IL-4 deprivation.

Endogenous IL-10 is an autocrinal factor for maturing DC suicide

The autocrine feedback of IL-10 potently inhibits the functionality of mature DCs, including their cytokine production and trafficking to the draining lymph node (26, 27). The secretion of endogenous IL-10 by maturing DCs may also play a role in determining their longevity. To examine the extent of influence on DC survival by endogenous IL-10, MDDC cultures were preincubated with anti-IL-10R1 mAb to block the IL-10 activity. Cells were activated with 10 ng/ml LPS, sufficient to promote IL-10 production by FACS-SSC and HLA-DR-CD86 to identify DCs. Numbers indicate frequencies of cells stained positive for active caspase-3. Results are representative of experiments from two different donors.

![Figure 1](http://www.jimmunol.org/) Exogenous IL-10 rapidly triggers apoptosis of LPS-activated DCs. MDDC cultures were stimulated with LPS in the presence or absence of hIL-10, cmvIL-10, or left untreated. Cells were stained with annexin V and PI (A) or active caspase-3 and assessed by FACS for apoptosis 12 h or 24 h after onset (B). A. Contour plots of annexin V (5%) and PI with outliers of cells after gating by FSC-SSC to identify DCs by size. Numbers in the lower right quadrants indicate percentages of cells undergoing apoptosis. The contour plot of unstained control is shown on the left. B. FACs for intracellular active caspase-3 after gating by FSC-SSC and HLA-DR-CD86 to identify DCs. Numbers indicate frequencies of cells stained positive for active caspase-3. Results are representative of experiments from two different donors.

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IL-10 limits the capacity of mature DCs to promote naive Th cell proliferation

The functional consequences of IL-10 exposure upon DC maturation were further demonstrated by the primary MLR assay. Differentially mature and immature MDDCs were collected at 48 h after treatment and cocultured with CFSE-labeled naive CD4+ T cells to evaluate their allostimulatory capacity. Exposure to exogenous IL-10 upon maturation severely impaired the capacity of DCs to promote naive CD4+ T cell proliferation (Fig. 2C), even though those DCs expressed comparable levels of MHC class II molecules (Fig. 4B). Conversely, when DCs were provided abundantly, the blockade of endogenous IL-10 activity did not change the capacity of LPS-matured DCs to activate allogeneic T cells. However, when DCs were less accessible to T cells in culture, the negative impact of endogenous IL-10 on their naive T cell priming capacity became noticeable (Fig. 2B). These data suggest that exogenous IL-10 exposure or autocrine IL-10 feedback leads to two outcomes: 1) lower numbers of mature DCs can make it to the draining lymph nodes; and 2) there is a shorter duration for the mature DCs to make contact with Ag-specific naive T cells.

Analysis of apoptosis-related gene expression regulated by IL-10

To investigate the molecular mechanisms of IL-10-induced DC apoptosis, a global gene expression profile of DCs was conducted by oligonucleotide arrays. MDDCs were treated with LPS ± either hIL-10 or cmvIL-10 for 12 h before RNA extraction. Because this study focused on the effects of IL-10 on phenotypically mature DCs, FACS was done upon aliquots of cells at 24 h posttreatment to confirm that >90% of cells exhibited the mature DC phenotype after LPS and IL-10 treatment.

The differential expression (DE) values of apoptosis-related genes, in comparison with untreated immature MDDCs, are summarized in Table I. Data analysis indicated that transcripts for ~2% of the unique genes exhibited either increased or decreased expression levels (DE ≥0.5 or DE ≤−0.5) triggered by IL-10 signaling. Three prosurvival genes among Bcl-2 family members, namely bcl-2, bcl-x, and bfl-1, were consistently suppressed by either hIL-10 or cmvIL-10 in mature DCs from both donors (Table I). The suppression of these genes in IL-10-treated DCs was coincident with their increased sensitivity to spontaneous apoptosis in culture. Array analysis also showed that both IL-10 molecules suppressed the expression of two other Bcl-2 family members, Bcl-G and NOXA. It is unlikely that the down-regulation of these two genes (the encodes of which promote apoptosis) was associated with increased apoptosis triggered by IL-10. It was also noted that IL-10 did not affect the expression of caspase family members (Table I).

Surprisingly, the global transcriptional profiles of mature DCs modulated by cmvIL-10 were very similar to those by host IL-10, despite the low amino acid sequence homology between these two molecules. This observation was consistent with the functional data conducted with both IL-10 molecules, which showed no measurable differences between hIL-10 and cmvIL-10 (Ref. 21 and Fig. 1). Therefore, hIL-10 was used to investigate the mechanism of IL-10-mediated DC apoptosis for the majority of remaining experiments.

IL-10 suppresses Bcl-2 accumulation associated with DC maturation

Because Bcl-2 was suppressed by hIL-10 and cmvIL-10 to the greatest extent (Table I), quantitative real-time PCR and immunoblotting assays were performed to evaluate the impact of IL-10 on the steady-state levels of Bcl-2 message and protein in DCs. The TLR family is the best-characterized class of receptors that recognizes pathogen-associated molecular patterns (PAMP). In addition to PAMPs, DCs can be activated by feedback signals from T cells and inflammatory mediators. We used LPS and sCD40L as stimuli, representing extrinsic and intrinsic DC activation signals, respectively, to study the kinetics of bcl-2 regulation by IL-10. The maturation of DCs was accompanied by a rapid accumulation of bcl-2 mRNA (Fig. 3A). The up-regulation of bcl-2 mRNA in DCs induced by CD40-mediated signaling was relatively short-lived and...
rapidly declined after 24 h of activation, compared with that induced by LPS-TLR4 ligation (Fig. 3A). IL-10 treatment did not abolish the increase of CD40L- or LPS-promoted bcl-2 mRNA in the cells, but markedly reduced it by ~80%. In two separate donors, IL-10 suppressed LPS-induced bcl-2 mRNA levels by 78 and 84%, respectively, 12 h posttreatment. Immunoblotting data showed that Bcl-2 in immature DCs was marginally above the level of detection. LPS and sCD40L induced 4.6- and 3.2-fold increases of Bcl-2 accumulation, respectively (Fig. 3B). IL-10 treatment reduced these increases by ~50% (54% for LPS and 52% for sCD40L; Fig. 3B). As the expression kinetics of bcl-2 mRNA (Fig. 3A) correlated with the accumulation of Bcl-2 protein (Fig. 3B), our data suggested that IL-10 regulates Bcl-2 expression in mature DCs, at least partially, at the transcriptional level.

We also conducted intracellular FACS to demonstrate the impact of IL-10 on Bcl-2 protein expression in the phenotypically
mature DCs. DCs were stained with mAbs specific for CD83 and HLA-DR to depict their maturation status (Fig. 4A, rectangular gates). In contrast to murine DCs, in which Bcl-2 protein is rapidly down-regulated after maturation (6), the level of Bcl-2 protein in mature human DCs was higher than that of immature DCs (Fig. 3A). Exogenous IL-10 treatment potently suppressed the up-regulation of Bcl-2 expression in LPS-matured DCs (Fig. 4B and C).

These data indicated that the lack of the Bcl-2 expression was attributable to IL-10 suppression in mature DCs, rather than to the inhibition of DC maturation. Ligands for other TLRs known to be expressed on MDDCs, LTA for TLR2 and poly(I:C) for TLR3, were also tested. Intracellular FACS indicated that Bcl-2 up-regulation in DCs triggered by these ligands was also suppressed by both hIL-10 and cmvIL-10 (Table II).

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** IL-10-mediated signaling inhibits the induction of bcl-2 gene expression. *A*, IL-10 suppresses the accumulation of bcl-2 mRNA within DCs activated by sCD40L or LPS. Total cellular RNA was extracted from MDDC cultures activated for 12 or 24 h in the presence or absence of recombinant hIL-10. Shown are the relative copy numbers of steady-state bcl-2 transcripts measured by quantitative real-time PCR analyses after normalization to GAPDH expression. Data are presented as the mean ± SD of triplicate assays. n.d., Not done. Statistic analysis between plus or minus hIL-10 samples of each treatment group was performed by Student *t* tests. **B**, Total cell lysates from MDDC cultures were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-Bcl-2 or anti-Bcl-xL Abs. Equal loading of bands was confirmed by Grb2 immunoblots. Shown are representative immunoblots of two separate experiments.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Extracellular signals dynamically control the Bcl-2 level in mature DCs. *A*, Up-regulation of Bcl-2 expression associated with DC maturation. *B*, IL-10 suppresses the up-regulation of Bcl-2 expression in phenotypically mature DCs. MDDC cultures were treated with poly(I:C), sCD40L or LPS in the presence or absence of recombinant hIL-10 for 24 h. Surface expression of CD83 and HLA-DR on DCs was assessed by FACS to define immature (CD83<sub>low</sub>HLA-DR<sub>low</sub>) and mature (CD83<sub>high</sub>HLA-DR<sub>high</sub>) DC populations, as indicated by boxes. Shown are 5% contour plots with outliers of cells after gating by FSC-SSC to identify DCs by size. Numbers represent percentages of the respective cell populations among total gated DCs. Intracellular Bcl-2 levels of respective DC populations are presented as histograms. Results are representative of five separate experiments. *C*, MFI data of Bcl-2 in unstimulated immature DCs or LPS-activated mature DCs under indicated treating conditions for 12 or 24 h. Dots in each color represent data from an individual donor (*n* = 3). Differences between groups were compared via Tukey’s multiple comparison tests. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001.

**Table II.** Bcl-2 expression of mature DCs treated with various ligands and IL-10

| Ligand | hIL-10 | cmvIL-10 |
|--------|--------|----------|
| LTA (5 μg/ml) | − | − | 100 100 |
|        | + | − | 68 68 |
|        | − | + | 56 79 |
| Poly (I:C) (50 μg/ml) | − | − | 100 100 |
|        | + | − | 50 55 |
|        | − | + | 56 52 |
| LPS (10 ng/ml) | − | − | 100 100 |
|        | + | − | 52 48 |
|        | − | + | 55 52 |
| sCD40L (1 μg/ml) | − | − | 100 100 |
|        | + | − | 64 66 |
|        | − | + | 57 60 |
| TNF-α (1000 U/ml) | − | − | 100 100 |
|        | + | − | 64 60 |
|        | − | + | 72 62 |

*MDDC cultures were treated with the indicated ligands in the presence or absence of 5 ng/ml of hIL-10 or cmvIL-10 for 24 h before assessed by FACS.*

*Expressed as percentage of the MFI in the absence of IL-10. Mature DC populations were identified by surface HLA-DR and CD83 expression as shown in Fig. 4.*
Endogenous IL-10 directs the kinetics of Bcl-2 expression in DCs

We next investigated whether the regulation of DC longevity by endogenous IL-10 was coincident with the alteration of bcl-2 expression in mature DCs. Intracellular FACS showed that endogenous IL-10 altered the Bcl-2 expression kinetics. The mean fluorescence intensity (MFI) data from two different time points of three different donors indicated that endogenous IL-10 exposure delayed and/or reduced Bcl-2 accumulation in maturing DCs. The mean expression in mature DCs. Intracellular FACS showed that endogenous IL-10 was coincident with the alteration of bcl-2 accumulation (data not shown).

Results are representative of two separate experiments. A. Levels of endogenous IL-10 in the supernatants measured by cytokine ELISA. n.d., Not done. Data are presented as the mean values ± SD of triplicate cultures. B. Steady state levels of bcl-2 or bcl-x mRNA within conditioned DCs. Shown are the relative copy numbers of transcripts measured by quantitative real-time PCR analyses after normalization to GAPDH expression. Real-time PCR data are presented as the mean ± SD of triplicate assays. The differences between the groups treated with isotype control mAb and the other two treatment groups were compared via the Dunnett multiple comparison test. *, p < 0.05; **, p < 0.01.

Impact of IL-10 on Bcl-xL expression

Array analysis also demonstrated that another important apoptotic regulator, Bcl-x, was suppressed by IL-10 (Table I), and this observation was confirmed by quantitative real-time PCR assay for bcl-x mRNA. Exogenous IL-10 treatment resulted in a reduction of bcl-x expression by an average of 50% in DCs activated with sCD40L, LPS, or poly(I:C) (Fig. 5B, sCD40L data not shown).

Alternate splicing results in two distinct isoforms of bcl-x mRNA, which encodes antiapoptotic Bcl-xL and proapoptotic Bcl-xS, respectively (31). Up-regulation of Bcl-xS has been reported to play an important role in protecting DCs from apoptosis upon activation by LPS and several TNF family members (7, 32). In murine DC11c−/− DCs, bcl-x mRNA is predominantly expressed as the long bcl-xL isoform (33). Immuno blotting was conducted to determine whether IL-10 influenced the accumulation of Bcl-xL in mature DCs, as neither microarray nor real-time PCR was designed to distinguish both isoforms. In contrast to Bcl-2, Bcl-xL protein in immature DC could be readily detected by immunoblotting, and LPS or sCD40L activation only led to relatively small increases (1.4-fold) of Bcl-xL (Fig. 3B). Exogenous IL-10 treatment reduced Bcl-xL protein expression by 10–15% in DCs activated through TLRs and by 24% in CD40L-activated DCs (Figs. 3B and 5C). We consistently observed the reduction of Bcl-xL accumulation caused by exogenous IL-10 exposure, even though the decrease level was relatively small, in contrast to that of Bcl-2 protein. Bcl-xL may still be an important target for IL-10 to regulate the longevity of DCs as rapid induction of Bcl-xL upon maturation is essential for the survival of mature DCs (9).

Although the up-regulation of Bcl-xL expression through TLRs was moderately sensitive to exogenous IL-10, it did not respond to autocrine IL-10 regulation. Blockage of endogenous IL-10 activity resulted in 2- to 3-fold increase of steady state bcl-x mRNA in maturing DCs (Fig. 5B). Surprisingly, this phenomenon did not
translate into a detectable increases of Bcl-xL protein accumulation in those DCs (Fig. 5C).

Modulation of Bcl-2 expression in freshly isolated PDCs and CD11c+ DCs by IL-10

CD11c+CD123− myeloid DCs and CD11c+CD123+ PDCs are the two major DC populations within human peripheral blood (2). Functionally, CD11c+ DCs are similar to MDDCs. They produce a variety of cytokines and chemokines and potently prime naïve T cells in response to PAMPS. PDCs are the main type I IFN producers among PBMCs in response to viral infection (34–36). IL-10 has been reported to induce the death of PDCs in vitro (23, 24). Therefore, we examined whether this is associated with the down-modulation of Bcl-2 expression. Peripheral blood CD11c+CD123− myeloid DCs and CD11c+CD123+ PDCs were isolated and assessed by intracellular FACS for Bcl-2. Based on the type of TLR expressed by these cells, poly(I:C) and CpG DNA were provided to stimulate CD11c+ DCs via TLR3 and PDCs via TLR9, respectively (Fig. 6). Exposure to exogenous IL-10 upon activation resulted in the suppression of Bcl-2 (average of 48% reduction in MFI) and CD86 expression of mature CD11c+ DCs (Fig. 6A, bottom). The scale of Bcl-2 reduction in poly(I:C)-activated CD11c+ DC caused by IL-10 was similar to that observed in MDDCs (Table II). For all four tested donors, we consistently observed a lower frequency of CD11c+ DCs in the IL-10-treated group (Fig. 6A, top), suggesting that IL-10 accelerated the loss of activated CD11c+ DCs in culture.

The responses of PDCs to IL-10 were distinct from those of myeloid DCs. Consistent with an earlier report (25), IL-10 had little effect on CD86 expression of CpG-activated PDCs (Fig. 6B, bottom). We also observed that IL-10 had a minor impact on Bcl-2 accumulation of CpG-activated PDCs (average of 27% reduction in MFI), suggesting differential regulation of PDC lifespan by IL-10. To further confirm the observed discordance of Bcl-2 expression between myeloid DCs and PDCs, their steady state bcl-2 mRNA was assessed by quantitative real-time PCR. Freshly purified CD11c+ DCs (A) and PDCs (B) were left untreated or treated with their responsive ligands in the presence or absence of recombinant hIL-10 for 16 h. The levels of bcl-2 mRNA were measured by quantitative real-time PCR analyses. Shown are the relative copy numbers of bcl-2 mRNA after normalization to GAPDH expression. Data are presented as the mean ± SD of triplicate assays. Statistic analysis between plus or minus hIL-10 samples of each stimulatory condition was performed by Student t tests. * p < 0.05; ** p < 0.01. Results are representative of two (CD11c+ DCs) or three (PDCs) separate experiments.

FIGURE 6. Responses of Bcl-2 expression to IL-10 in blood DCs. Freshly isolated blood DCs were treated with either poly(I:C) (A) or CpG (B) in the presence or absence of recombinant hIL-10 for 16 h. Surface expression of CD11c and CD123 was assessed by FACS to identify each population as indicated (top). Shown are 5% contour plots with outliers of cells after gating by FSC-SSC to identify DCs by size. Numbers represent percentages of the respective cell populations among total gated cells. Shown in bottom panels are overlaid histograms of surface CD86 and intracellular Bcl-2 expression of poly(I:C)-activated CD11c+ DCs (A) and CpG-activated PDCs (B). Results are representative of isolated DCs from four different donors.

FIGURE 7. Regulation of bcl-2 transcription in primary PDCs and CD11c+ DCs. Freshly purified CD11c+ DCs (A) and PDCs (B) were left untreated or treated with their respective ligands in the presence or absence of hIL-10 for 16 h. The levels of bcl-2 mRNA were measured by quantitative real-time PCR analyses. Shown are the relative copy numbers of bcl-2 mRNA after normalization to GAPDH expression. Data are presented as the mean ± SD of triplicate assays. Statistic analysis between plus or minus hIL-10 samples of each stimulatory condition was performed by Student t tests. * p < 0.05; ** p < 0.01. Results are representative of two (CD11c+ DCs) or three (PDCs) separate experiments.
up-regulation of bcl-2 mRNA in CpG-activated PDCs by an average of 35% (range, 28–43%; Fig. 7B). Similar patterns of bcl-2 mRNA reduction caused by IL-10 were seen in PDCs activated through TLR7 by imiquimod or heat-inactivated influenza virus (Fig. 7B).

Discussion

The abundance and longevity of migrant Ag-bearing DCs in secondary lymphoid tissues are the key factors in determining the magnitude of the adaptive immune responses (5, 6, 37). DC homeostasis and natural turnover must be appropriately orchestrated to ensure the generation of protective T cell responses on one hand and to prevent the immunopathological outcomes on the other. In this study, we show that both externally added and activation-induced autocrine IL-10 production alter the lifespan of mature myeloid DCs. The mechanism behind that is likely associated with the dynamic regulation of antiapoptotic Bcl-2 proteins expression by IL-10.

Bcl-2 and Bcl-xL are two key anti-apoptotic components that regulate the longevity of DCs (9). Extending from the previous findings, our data suggest a model of Bcl-2 and Bcl-xL expression by DCs in relation to the exposure of IL-10 (Fig. 8). bcl-2 expression was up-regulated in human myeloid DCs activated by signals initiated through TNFR family members or TLRs, an observation that stands in contrast to the observations made in murine DCs, in which the Bcl-2 message and protein is rapidly diminished upon DC maturation (6, 38, 39). Although the mechanism remains to be determined, there is differential regulation of bcl-2 expression by different activation signals in DCs. CD40L-induced bcl-2 expression is rather short-lived, compared with that induced by LPS. Sporri and Reis e Sousa (40) reported that PAMP-activated mature DCs are licensed to support CD4+ T cell differentiation into effector helper cells whereas inflammatory mediators only enable DCs to induce the proliferation of naive T cells. Whether this is attributable to the sustenance of bcl-2 expression in mature DCs is currently under investigation.

The effect of exogenous IL-10 on maturing DCs was rapid and sustained. IL-10 severely attenuated the increase in bcl-2 transcription after bcl-2 transcription after LPS, poly(I:C), or CD40L activation with a subsequent decrease in Bcl-2 protein expression. In contrast to the active modulation of Bcl-2 by IL-10, Bcl-xL was less labile to IL-10-mediated modulation. We showed that CD40L, LPS, and poly(I:C) induce similar levels of Bcl-xL protein accumulation in the mature DCs, which are moderately sensitive to exogenous IL-10 down-regulation. Our data suggest that, when myeloid DCs exposed to exogenous IL-10 upon activation, the deficit of both Bcl-2 and Bcl-xL is associated with their rapidly reduced lifespan. Consistent with our finding, DCs from transgenic mice constitutively expressing Bcl-2 are present in greater numbers and have a prolonged survival in vivo (6). Conversely, DCs from bcl-2-deficient mice have a reduced survival compared with controls (9). Another study showed that bcl-xL-deficient DCs fail to mount effective immune responses resulting from their rapid disappearance from the draining lymph node due to apoptosis (33).

TLR engagement triggers secretion of pro-inflammatory cytokines by mature DCs (10) and, on the other hand, promotes IL-10 production to down-modulate these responses. Mature DCs are no longer sensitive to the inhibitory effects of IL-10 (41–43), due to the loss of their surface IL-10R1 and IL-10 binding activity after DC maturation (27). We found that endogenous IL-10 acts as a suicidal factor for myeloid DCs in an autocrine fashion. The engagement of endogenous IL-10 to its receptor gradually decreased the vial LPS-induced DC numbers in culture, in contrast to the culture treated with anti-IL-10R1 mAb. This phenomenon is contemporaneous with the altered kinetics of Bcl-2 expression in maturing DCs triggered by endogenous IL-10. The blockade of endogenous IL-10 activity resulted in enhanced transcription for both bcl-2 and bcl-xL. Interestingly, the immunoblotting data showed that autocrine IL-10 impacts the steady state protein level only of Bcl-2, not Bcl-xL. Whether posttranscriptional regulation is essential for further accumulation of Bcl-xL protein at the later stage of DC maturation, similar to the manner observed in keratinocytes (44), remains to be studied. Taken together, our data demonstrate that the scale of Bcl-2 expression in myeloid DCs is dynamically modulated by the degree of IL-10 exposure before DCs become insensitive to IL-10 engagement. In contrast, Bcl-xL expression is shaped when immature DCs receive extracellular signals initially and does not contribute to the subsequent regulation of DC homeostasis.

Cell apoptosis is also regulated by other Bcl-2 family members, including both pro- and antiapoptotic proteins that share up to four conserved regions termed Bcl-2 homology (BH) 1–4 domains (45). Array analysis also indicates that IL-10 modulates the expression of another pro-survival Bcl-2 family member, Bfl-1/A1. Bfl-1/A1 expression is generally confined to immune cells/tissues, and it suppresses apoptosis triggered by TNF-α, BCR aggregation, and proapoptotic factors Bax and Bad (46–50). Constitutively elevated levels of bfl-1 transcripts are seen in mature monocytes and are selectively induced in long-lived peripheral B cells (49, 50). Similarly, in LPS-activated mature DCs, we also found a marked
increase of bfl-1 mRNA by microarray analysis, and this induction was moderately suppressed by IL-10. The results of array analysis suggest that IL-10 has little effect on the expression of other Bcl-2 family members. Bid is a BH3-only molecule that binds proapoptotic Bax and Bak, as well as antiapoptotic Bcl-2 and Bcl-xL (51, 52). Bcl-2 or Bcl-xL sequester BH3 domain-only proteins in stable mitochondrial complexes and therefore prevent Bax and Bak activation (53, 54). We have investigated the steady state levels of Bax and Bid in CD40L- or LPS-activated DCs by immunoblotting. In agreement with our microarray data, IL-10 treatment did not alter their expression profiles in mature DCs (data not shown).

The suppression of Fas up-regulation in mature DCs by IL-10 was also detected in microarray data. Unlike B cells and macrophages, DCs or monocytes are not susceptible to Fas-induced cell death (55–59). Fas is unable to induce DC death due to the constitutive expression of FLIP (55, 56), an important apoptosis-regulatory protein that interferes with the activation of caspase-8 (60). Raftery et al. (20) recently reported that IL-10 moderately blocks the up-regulation of the antiapoptotic long form of FLIP expression in LPS-activated DCs, which also plays an important role in promoting apoptosis of IL-10-treated DCs.

IL-10 has been shown to cause the reduction of PDCs numbers in vitro (23, 61). The mRNA and protein data indicated that IL-10 moderately suppresses the up-regulation of Bcl-2 expression in PDCs, in contrast to myeloid DCs, suggesting differential regulation of PDC survival by IL-10. IFN-α is a strong autocrine PDC survival factor (34), and stronger IFN-α induces protect PDCs from IL-10-promoted rapid cell death (25). Because IL-10 also suppresses type I IFN production of mature PDCs (W. L. W. Hou, L. Van Parijs. 2004. A Bcl-2-dependent molecular timer regulates the lifespan and immunogenicity of dendritic cells. Nat. Immunol. 5: 583–589.

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