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*J Immunol* 2006; 177:8550-8559; doi: 10.4049/jimmunol.177.12.8550

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Semimaturity Stage: A Checkpoint in a Dendritic Cell Maturation Program That Allows for Functional Reversion after Signal-Regulatory Protein-α Ligation and Maturation Signals

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CD47 on live cells actively engages signal-regulatory protein-α (SIRP-α) on phagocytes and delivers a negative signal that prevents their elimination. We evaluated the biological consequences of SIRP-α ligation on the dendritic cell (DC) response to maturation signals and the potential interplay with the IL-10/IL-10R inhibitory pathway. At first, CD47/SIRP-α allowed the generation of mature migratory DCs not producing IL-12, IFN-γ-inducible protein-10, and CCL19. Rather, they secreted neutrophils attracting chemokine CXCL5 and IL-1β, reflecting a partial block in functional DC maturation. Afterward, semimature DCs functionally regressed in an IL-10-independent fashion toward cells that retrieved the cardinal features of immature DCs: re-expression of CCR5, loss of DC-lysosome-associated membrane protein, high endocytosis, and impaired allostimulatory functions. The global gene expression profile of IL-10 and SIRP-α-ligated DC demonstrated two distinct molecular pathways. IL-10R and SIRP-α expression were reciprocally down-regulated by CD47 and IL-10, respectively. These results emphasize that the SIRP-α pathway might be part of the molecular machinery used by the DC to dampen or resolve an inflammatory response in an IL-10-independent manner. The Journal of Immunology, 2006, 177: 8550–8559.

Dendritic cells (DC) are professional APCs, unique in their ability to activate naive T cells. They “patrol” peripheral tissues, sample autoantigens as well as pathogens, and play an essential role in the control of both tolerance and immunity (1, 2). Upon encounter with a pathogen or an inflammatory signal, immature DC (iDC) lose their phagocytic and Ag processing capacities and become efficient APCs characterized by their secondary lymphoid organ migratory tropism and enhanced cytokine and costimulatory molecule expression. DC maturation-induced shift in migratory properties is believed to be the consequence of a switch in chemokine receptor surface expression. Indeed, maturing DC down-regulate chemokine receptors which would drive their migration toward inflammatory sites (such as CCR1, CCR2, and CCR5) and concomitantly up-regulate expression of CCR7, a chemokine receptor which drives their migration toward lymphoid organs along MIP-3β (CCL19) and 6Ckine (CCL21) chemokine gradients (3, 4).

Although many aspects of DC-mediated priming of T and B lymphocytes within lymphoid organs have already been extensively studied, how DC contribute to the resolution phase of an adaptive immune response remains controversial. It is possible that reduction in the abundance of Ag-loaded mature DC through passive or active killing mechanisms might contribute to the immune response termination. Alternatively, production of suppressive mediators, such as IL-10 and TGF-β, might contribute to desensitizing both APCs and lymphocytes in the late phase of the immune response and render them refractory to subsequent stimulation (5–7).

IL-10 is a potent inhibitor of DC maturation. It prevents both differentiation of myeloid progenitors into iDC and their subsequent differentiation into APCs (6, 8). Hormones such as 1,25 dihydroxy-vitamin D₃ or 17β-estradiol also suppress DC differentiation and maturation both in vitro and in vivo (9, 10). In addition to those soluble factors, we reported that engagement of surface receptors, such as signal-regulatory protein-α (SIRP-α), down-regulates DC maturation (11).

SIRP-α (also known as CD172a, SHPS-1, BIT, MYD, or p84) is a type I transmembrane receptor of the Ig superfamily expressed on DC as well as many other leukocytes of the myelomonocytic lineage (monocytes, macrophages, neutrophils). SIRP-α is expressed in neurons, skeletal muscle cells and endothelial cells. Its intracellular domain contains two ITIM as well as two immunoreceptor tyrosine-based switch-like motifs which recruit Src homology region 2 domain-containing phosphatase-1 and -2 thus inhibiting signaling through various receptor tyrosine kinases and cytokine receptors (12). SIRP-α has been involved in as many phenomena as suppression of anchorage-independent cell growth, mediation of macrophage multinucleation, skeletal muscle differentiation, neuronal survival, and synaptogenesis.

Received for publication April 5, 2006. Accepted for publication October 3, 2006.

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3Abbreviations used in this paper: DC, dendritic cell; iDC, immature DC; SIRP, signal-regulatory protein; nDC, mature DC; SAC, Staphylococcus aureus Cowan I strain; SDP, stromal cell-derived factor; LN, lymph node; IP-10, IFN-γ-inducible protein-10; LAMP, lysosome-associated membrane protein.
The CD47 receptor, an ubiquitously expressed multispanning transmembrane protein, and lung collectins, serve as ligands for SIRP-α (14–17). CD47 is considered to be a marker of self on hematopoietic cells. Its interaction with SIRP-α prevents the elimination and clearance of RBC and mononuclear cells by macrophages (18, 19). By contrast, CD47 expression on apoptotic cells is absolutely required for their engulfment by APCs, suggesting that CD47/SIRP-α interaction works as a tethering step in phagocytosis (20). Finally, CD47/SIRP-α interaction modulates neutrophil transendothelial and transepithelial migration (21). However, the details about how and on which cell type CD47 interacts with SIRP-α to regulate neutrophils migration remain to be determined (22).

In this report, we focused on the cellular and molecular consequences of SIRP-α engagement on DC and studied the potential interplays between IL-10/IL-10R and CD47/SIRP-α pathways. We show that SIRP-α ligation induced an IL-10-independent functional block in DC maturation and migration in response to danger signals. This molecular brake promoted the generation of “semi-mature” DC that, at a later phase of their maturation process, acquired the cardinal features of immature-like cells. Gene expression profiling revealed that SIRP-α-triggered DC, but not IL-10-exposed DC, expressed several neutrophils attracting chemokines and IL-1 family members. Semimaturity may be thus be considered as the initial step of a two-step DC maturation program, a checkpoint controlled by CD47/SIRP-α interactions that allows for functional reversion.

Materials and Methods

Cell preparation and culture conditions

Human PBMC and iDC were prepared as described (23, 24). Mature DC (mDC) were generated following stimulation of iDC (1 × 10⁶/ml) for the time indicated in the figures with Staphylococcus aureus Cowan I strain (SAC) at 1/40,000 (Pansorbin; Calbiochem-Behring), LPS (10 μg/ml) in complete HB101 medium (Irvine Scientific). Soluble CD47-Fc (prepared and used as described in Ref. 11) was added at 10 μg/ml. Briefly, CD47-Fc is composed of the extracellular domain of CD47 (aa 1–142) fused to a mutated human IgG1 Fc region to lower the binding to FcRs; it was used either in soluble form with SAC or immobilized on plastic-coated goat anti-human Ig (Tago, BioSource International) with LPS. The soluble Fc fragment from normal human Ig (NHg-Fc) or the FOX-40 Fc fusion molecule (a molecule that binds to DC and contains the same Fc portion as CD47-Fc) were used as control (referred in the text as Ctrl-Fc). No difference was ever observed between SAC/LPS alone, SAC/LPS plus NHg-Fc, or SAC/LPS plus FOX-40-Fc in our experiments. IL-10 (used at 20 ng/ml) was a gift from DNAX (Palo Alto, CA), PGE2 (1 μg/ml) was purchased from Sigma-Aldrich, and the anti-IL-10-mAb (10 μg/ml) was produced in the laboratory (American Type Culture Collection (ATCC) cell line HB10487, clone 19F111). The recovery of viable cells was assessed by trypsin blue dye exclusion or Annexin V-FITC staining.

Highly purified T cells were obtained from monocyte-depleted PBMC by rosetting with 2-aminooethylisothiouronium bromide-treated sheep, followed by treatment of rosette-forming cells with Lympho-Kwik T (One Lambda) according to the manufacturer’s recommendations. Cell purity was assessed by flow cytometry using PE-conjugated anti-CD3 (Ancell) and was shown to be >98%. MRls were conducted in 96-well flat-bottom microtiter plates (Falcon; BD Biosciences) for 5 days in complete culture medium (RPMI 1640 10% CS). iDC were cultured for 24 or 48 h in the absence or presence of SAC with CD47-Fc or control fusion protein, washed, and then added to T cells (10⁶/ml) at various stimulator (DC)/responder (T cell) ratios. [3H]Thymidine was added during the last 6 h of the culture. Half of the culture supernatant was removed previously to tinylated nucleoside triophosphate by means of a BioArray High Yield washing using cold PBS containing 1% BSA and 10 mM sodium azide before analysis on FACSCalibur.

Flow cytometry analysis

CD86, CD83, CCR5 (clone 2D7), CXCXR4, CD172 (SE5A5), and DC-lysosome-associated membrane protein (LAMP) were purchased from BD Biosciences. Anti-CD172a (clone mSIRP30) was a gift from A. Oldenborg (St. Louis, MO). The anti-HLA-DR mAb was purified in the laboratory from culture supernatant (ATCC HB104) and labeled with the Alexa 488 kit (Molecular Probes). The CCR7 (BD, clone 2H4) staining was followed by FITC-goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Anti-IL-10R mAb (clone 3F9; BD Biosciences) staining was revealed with a biotinylated goat anti-rat IgM plus G mAb (Southern Biotechnology Associates) followed by PE-coupled streptavidin (BD Biosciences). Stained cells were analyzed using a FACS Calibur (BD Biosciences).

Confocal microscopy

For intracellular immunofluorescence staining, cells were incubated at 37°C for 20 min on polylysine-coated coverslips before fixation for 15 min with 4% paraformaldehyde in PBS. Coverslips were then washed twice in 10 mM glycine in PBS and twice in PBS and permeabilized with 0.5% saponin, 1% BSA in PBS for 30 min. They were incubated for 30 min at room temperature with 5 μg/ml anti-DC-LAMP (Immunotech) or anti-HLA-DR in permeabilization medium and revealed by incubation for 30 min with 2 μg/ml secondary Ab (Alexa Fluor 594 or Alexa Fluor 546, goat anti-mouse IgG; Molecular Probes). Coverslips were blocked with mouse preimmune serum for 30 min. For double labeling, they were next incubated for 30 min with either anti-HLADR or anti-CD86 Ab directly coupled to Alexa 488 or FITC, respectively. Coverslips were mounted onto glass slides with Vectashield mounting medium (Vector Laboratories). Confocal microscopy was performed using the Zeiss Axiovert 100TV ConfoLaser Microscope and the LSM 510 module with Ar (458, 488, and 514 nm), HeNe (543 nm), and HeNe (633 nm) lasers (Carl Zeiss). Images were acquired with the LSM5 image program (version 2.8).

Cytokines and chemokines measurement

IL-12/70, IL-10, TNF-α, and IFN-γ release were assessed by a two-site sandwich ELISA (24). The MIP-3β, MIG, IFN-γ-inducible protein-10 (IP-10), IL-6 ELISA kit were purchased from BD Biosciences and CXCL5, CCL1, CCL13, and IL-1 from R&D Systems. All the measurements were performed in duplicate.

In vitro migration assay

In vitro DC migration was performed using a 96-well microchemotaxis chamber (Neuroprobe; Chemotx 101-5). After determination of optimal concentration by serial dilution of chemokines, 30 μl of stromal cell-derived factor 1 (SDF-1) or MIP-3β (PeproTech) at 100 ng/ml in HBSS 0.1% BSA were added to the lower chamber. A polycarbonate-filter (0.5 μm) was layered onto the wells. On top of the filter, 4 × 10⁵ cells were added to 2 μl of HBSS 0.1% BSA. After incubation at 37°C, 5% CO₂ for 2 h, cells were removed by aspiration and replaced by a solution of EDTA 2 mM in PBS. Plates were incubated for 30 min at 4°C. EDTA was eliminated, and the plates were centrifuged for 5 min at 1400 rpm. The filter was removed and 50 μl of medium plus 40 μl of a solution of 10-μm polystyrene latex beads (Beckman Coulter) at 5 × 10⁵ beads/ml was added to the migrated cells. The whole volume was transferred to Titerplates (Bio-Rad). The number of migrated cells, represented either as the raw data or after subtraction of the background (medium without chemokine), was evaluated by counting fixed amount of beads using FACS Calibur.

Microarrays and data analysis

Human genome-wide gene expression was examined using the Human Genome U133A probe array (GeneChip; Affymetrix) that contains the oligonucleotide probe set for ~22,000 full-length genes, according to the manufacturer’s protocol. Total RNA was extracted from ~5 × 10⁶ cells. Briefly, iDC were stimulated (in HB101 supplemented with polyoxymycin B for 16 h in the absence or presence of Ctrl-Fc, CD47-Fc, IL-10, SAC, LPS (10 μg/ml) plus IL-10, LPS plus IL-10, SAC plus CD47-Fc, and LPS plus CD47-Fc. The samples were prepared from six different healthy donors and pooled before total RNA extraction. Double-stranded cDNA was synthesized by means of SuperScript Choice system (Invitrogen Life Technologies) and a T7-25234 primer (Amersham Biosciences). The cDNA was subjected to the in vitro transcription in the presence of biotinylated nucleotide triphosphate by means of a BioArray High Yield
RNA Transcript Labeling kit (Enzo Diagnostics). The biotinylated cRNA was hybridized with a probe. After washing, the hybridized biotinylated cRNA was stained with PE-streptavidin (Molecular Probes) and then scanned with an HP Gene Array Scanner (Affymetrix). The fluorescence intensity of each probe was quantified with a computer program, Suite 4.0 (Affymetrix).

For two-dimensional cluster representation, an ordered list of ratio experiments were clustered with preselected sequences using Rosetta Resolver gene expression data analysis system. Sequences were clustered using an agglomerative algorithm with average heuristic link and error weight Pearson type correlations. A two-dimensional heat map was then constructed using Spotfire.

Results

**CD47/SIRP-α interaction led to IL-10-independent interruption of DC maturation**

CD47 on live cells actively engages SIRP-α on phagocytes and counteracts the “eat me” signal provided by calreticulin/CD91 interactions (25). We have previously shown that SIRP-α ligation by CD47-Fc fusion protein inhibits both cytokine secretion and typical surface markers induction on maturing DC (11). Because autocrine IL-10 exerts comparable effects on maturing DC (26), we here designed experiments to evaluate how SIRP-α triggering and IL-10 exposure alter the complete DC maturation program in response to danger signals and assessed the contribution of endogenous IL-10 in SIRP-α-mediated inhibition of DC maturation.

DC were exposed to SAC particles to induce maturation in the presence or absence of CD47-Fc or IL-10. The effect of IL-10 vs CD47-Fc on DC maturation was assessed in a 48-h time course experiment by monitoring MHC class-II (HLA-DR) translocation and DC-LAMP expression by confocal microscopy (Fig. 1A). As previously described, DC translocated MHC class II molecules from intracytoplasmic compartments to the cell surface (27) and concomitantly up-regulated DC-LAMP (28) within 9 h following SAC stimulation. In the presence of IL-10, MHC class II molecules did not translocate to the cell surface at any time point examined. Only sporadic expression of DC-LAMP was observed in intermediate compartments where it colocalized with HLA-DR. By contrast, SIRP-α engagement on maturing DC did not significantly alter the kinetics of SAC-induced HLA-DR translocation up to 24 h of culture. At that stage, DC exhibited an intermediate phenotype characterized by surface MHC class II and perinuclear DC-LAMP expression. At 48 h, most of the DC cultured in the presence of CD47-Fc displayed an immature phenotype comparable to that observed at the culture initiation, suggesting that
SIRP-α stimulation led to phenotypic reversion from an intermediate to an immature DC phenotype between 24 and 48 h of culture.

The existence of an intermediate phenotype at 24 h and the reacquisition of an immature phenotype between 24 and 48 h of culture were further substantiated by flow cytometric analysis (Fig. 1, B and C). After 24 h, cells engaged through SIRP-α expressed less CCR7, CD83, CD86, and HLA-DR than mDC but significantly more than iDC. In addition, expression of CCR5, a chemokine receptor down-regulated during DC maturation, was slightly but significantly increased on CD47-treated DC when compared with SAC-stimulated DC. Triple-labeling experiments demonstrated that the proportion of DC-LAMP<sup>−</sup>CD86<sup>high</sup>CCR5<sup>low</sup> cells within the CD47-Fc-treated DC population increased up to 24 h and subsequently decreased to reach basal iDC expression levels at 48 h (Fig. 1C). At that time, SIRP-α-stimulated cells and iDC displayed similar phenotype as assessed by CD83, CD86, HLA-DR, CCR5, and CCR7 surface expression. As a control, no significant difference in viable cells recovery was observed between CD47-Fc-treated and untreated cultures indicating that phenotypic reversion between 24 and 48 h is not the result of selective death of DC with intermediate phenotype. Of note, phenotypic reversion was also observed when CD47-Fc was added before or up to 2 h after SAC stimulation (data not shown).

Anti-IL-10-neutralizing Ab overcame the IL-10 inhibition of DC maturation (data not shown) with no effect on the phenotypic reversion induced by CD47-Fc suggesting that SIRP-α and IL-10 mediate inhibition of DC maturation through independent pathways (Fig. 1D). Consistent with this notion, CD47-Fc readily suppressed IL-10 production as early as 24 h after activation (Table I). The secretion of proinflammatory cytokines and chemokines was abrogated in SIRP-α and IL-10-stimulated DC.

Thus, although both CD47-Fc and IL-10 interfere with the in vitro DC maturation program, those two factors showed significantly different modes of action: IL-10 blocked DC maturation whereas CD47-Fc first induced DC to proceed through an intermediate stage of maturation in an IL-10-independent fashion yielding to semimature DCs (29, 30). Then, SIRP-α ligation induced the final differentiation of semimature DC to immature-like cells.

**CD47/SIRP-α interaction mediated transient DC migration in an IL-10-independent manner**

During a complete maturation program, DC acquire the expression of the chemokine receptor CCR7 and migrate toward its ligands, CCL19 and CCL21. CXCR4, the receptor for CXCL12, is another chemokine receptor whose expression is reportedly increased upon DC maturation (3). We next thought to evaluate whether CD47-Fc treatment could also impair the chemotactic response to CCL19 (MIP 3β) and CXCL12 (SDF-1) at different stages of maturation (Fig. 2A). After 24 h of culture in the presence of CD47-Fc, semimature DC expressed CCR7 and CXCR4 and migrated efficiently toward CCL19 and CXCL12 as assessed by microchemotaxis chamber assays (Fig. 2, A and B). After 48 h of culture, SIRP-α-stimulated DC showed reduced CCR7 and increased CXCR4 surface expression levels when compared with mature DC and failed to migrate toward either CCL19 or CXCL12. Thus, surface expression of CXCR4 did not correlate with the SIRP-α-stimulated DC chemotactic response to CXCL12 gradient. Interestingly, CXCR4 up-regulation occurred as soon as 4–6 h postengagement of SIRP-α with or without a concomitant maturation signal; this up-regulation was inhibited by brefeldin A but not cycloheximide (D. Braun and M. Sarfati, unpublished data).

Several publications have evidenced the role of PGE<sub>2</sub> as a critical modulator of DC migration (31, 32). It was thus essential to examine whether the presence of exogenous PGE<sub>2</sub> overcame the inhibition of DC migration observed 48 h after SIRP-α ligation. The data in Fig. 2C clearly indicate that CD47-Fc inhibited DC chemotaxis even in the presence of PGE<sub>2</sub>. PGE<sub>2</sub> strongly enhanced the chemotaxis of mDC as well as the expression of the chemokine receptors CCR7 and CXCR4. However, CD47-Fc inhibited CCR7 and still up-regulated CXCR4 even in the presence of PGE<sub>2</sub> (Fig. 2C). Similarly, in the presence of PGE<sub>2</sub>, aborted DC expressed low amounts of CD86, comparable to those of iDC. Thus, the inhibition of mDC chemotaxis following SIRP-α engagement was likely not to result from PGE<sub>2</sub> deprivation.

Finally, IL-10 efficiently inhibited DC chemotaxis but neutralizing IL-10 did not prevent the loss of migration induced by SIRP-α ligation, further demonstrating that IL-10 and CD47 independently inhibited DC chemotaxis (Fig. 2D).

**CD47/SIRP-α interaction promoted the alternate differentiation of semimature DC toward functional immature-like DC**

The transition from high to low endocytic capacity characterizes in vitro DC maturation and is believed to reflect in vivo progression from an Ag capture to an Ag-presenting state. We have shown that, in response to SIRP-α ligation, DC reach a semimature stage before regression toward ‘immature like’ stage of differentiation. It was therefore important to examine whether reacquisition of an immature phenotype would also encompass recovery of endocytic capacity.

As expected, whereas immature DC were potent endocytic cells capable of internalizing fluorescein-coupled BSA (Fig. 3A), after 24 h maturation in the presence of SAC, maturing DC...
displayed a greatly reduced endocytic activity. Likewise, when cultured in the presence CD47-Fc for 24 h, maturing DC showed decreased BSA uptake. In contrast, after 48 h of culture with CD47-Fc, SAC-stimulated DC were capable of endocytosis. Therefore, SIRPα-induced functional reversion extends to the main functional characteristics of iDC, namely, their ability to endocytose Ags.

The mDC are refractory to secondary stimulation in that they produced less proinflammatory cytokines than iDC following re-stimulation by SAC or CD40L plus IFN-γ, a factor combination that mimics the T cell-dependent signals (33). We tested whether CD47-Fc stimulation would also result in restoration of cytokine production capacity. After 48 h stimulation, DC were harvested

The mDC are refractory to secondary stimulation in that they produced less proinflammatory cytokines than iDC following re-stimulation by SAC or CD40L plus IFN-γ, a factor combination that mimics the T cell-dependent signals (33). We tested whether CD47-Fc stimulation would also result in restoration of cytokine production capacity. After 48 h stimulation, DC were harvested
and subjected to a secondary stimulation with SAC or CD40 plus IFN-γ. Fig. 3B shows that, after 48 h in the presence of CD47-Fc, SAC-stimulated DC recovered their ability to secrete TNF-α in response to a secondary T cell-dependent stimulation. Finally, we examined the T cell stimulatory function of immature-like CD47-Fc-treated DC in mixed lymphocyte cultures. DC were matured in the presence or absence of CD47-Fc, washed and cocultured with adult CD4+ T cells for 5 days. As expected mDC, but not iDC, strongly stimulated T cell proliferation and IFN-γ production (Fig. 3C). SIRP-α-stimulated immature-like DC displayed low T cell allostimulatory capacity (Fig. 3, C and D).

Taken together, these data show that, upon prolonged SIRP-α stimulation, maturing DC retrieve cardinal functional attributes of immature DC, including 1) high endocytic capacity, 2) responsiveness to secondary stimulation, and 3) poor allostimulatory activity.

**SIRP-α ligation induced an exclusive gene expression profile that reflects a partial block in DC maturation**

We have shown that CD47/SIRP-α and IL-10/IL-10R interactions initiate two independent regulatory processes that antagonize human DC maturation induced by proinflammatory signals. As such, both interactions might constitute attractive therapeutic intervention points for the treatment of autoimmune and inflammatory diseases. One major limitation in our understanding of IL-10 and SIRP-α-mediated regulation of human DC maturation in vivo lies in the fact that no specific biomarkers of activity have been identified for SIRP-α or IL-10R agonists. We therefore examined the global gene expression profile of the CD47-Fc-treated DC and compared it to that induced by IL-10 either in the absence or presence of maturation signals. Briefly, iDC from six independent healthy donors were stimulated for 16 h in the presence or absence of maturation agents (SAC or LPS) and simultaneously treated with either control fusion protein, CD47-Fc or IL-10. The culture supernatants were collected to assess cytokine and chemokine production and DC were collected for mRNA extraction. Fig. 4A shows SAC-stimulated DC genes that displayed differential expression (p < 0.005) in response to CD47-Fc and/or IL-10. Although IL-10 and CD47-Fc exhibited a common profile of 686/702 (up-/down-regulated) genes, we concentrated our analysis on those genes that appear exclusively regulated by IL-10 (1746/1758 red) or CD47-Fc (934/942 green), as well as counterregulated genes (62/63 purple). Only 37 genes were up- or down-regulated by 3-fold in response to CD47-Fc in SAC-stimulated DC (data not shown). The most up-regulated genes (≥5-fold, p < 0.005) included three genes encoding for CXCR2-ligands (i.e., CXCL5, CXCL6, and CXCL7 (PPBP)), CCL1, and CLEC5F/MDL-1 (a member of c-type lectin-like domain superfamily associated with DAP-12) (34). Conversely, legumain (asparaginyl endopeptidase...
(AEP), a lysosomal cysteine protease expressed in DC (35)) and P8 (nuclear protein 1, a gene involved in the response to pro-apoptotic stimuli and promotion of cell growth) were down-regulated by CD47-Fc but not by IL-10. The gene expression of the IL-1 family members were inversely correlated in SIRP-\(\alpha\)/H9251 and IL-10-stimulated DC (Table II). Note that CXCL5, legumain, and P8 genes were regulated by CD47-Fc regardless of the DC maturation status and the quality of the stimulus. In Fig. 4B, genes that showed differential expression (\(p < 0.005\)) in response to either CD47-Fc or IL-10 are shown. IL-10 and CD47-Fc-regulated genes appear in red and green, respectively. Coregulated genes appear in yellow whereas antiregulated genes are purple. Genes that also appear in Table II and panel B are highlighted.

FIGURE 4. CD47 and IL-10 induce distinct and different gene expression profiles. Human monocyte-derived DC from six separate donors were left unstimulated or matured for 16 h as indicated: (SAC (1/40,000) or LPS (1 \(\mu\)g/ml) in the presence of control Ctrl-Fc (medium), CD47-Fc (10 \(\mu\)g/ml), or IL-10 (20 ng/ml). Cells were pooled before RNA extraction. A, Comparison plot of IL-10 vs CD47-Fc regulated genes in SAC-activated DC. Genes that show differential expression (\(p < 0.005\)) in response to either CD47-Fc or IL-10 are shown. IL-10 and CD47-Fc-regulated genes appear in red and green, respectively. Coregulated genes appear in yellow whereas antiregulated genes are purple. Genes that also appear in Table II and panel B are highlighted. B, DC genes differentially regulated by IL-10 and CD47-Fc. Genes showing differential expression (\(-3 < \text{fold change} > 3; p < 0.005\)) after in vitro treatment of immature or mature DC by either IL-10 or CD47-Fc were first selected. Genes that were up- or down-regulated by both IL-10 and CD47-Fc in the absence or presence of SAC or LPS were then discarded. The remaining 27 genes were selected for this analysis and were clustered on the basis of their regulation. The degree of redness and greenness represent induction and repression, respectively. Variations in genes expression in this final list were comprised between \(-17-\) and \(+31-\)fold. Genes whose expression was regulated by \(<2\)-fold appear in black.

C, DC from eight separate donors were matured for 16 and 48 h as indicated. Chemokines and cytokines production was measured in the culture supernatant by ELISA. Mean \(\pm\) SEM of eight experiments.

Finally, CD47 and IL-10 not only regulated a differential set of genes but reciprocally down-regulated IL-10R and SIRP-\(\alpha\) in both iDC and mDC. In fact, CD47/SIRP-\(\alpha\) strongly suppressed IL-10R expression and CD47-Fc-treated DC expressed even lower amounts of IL-10R than iDC (Fig. 5A). Conversely, IL-10 down-regulated SIRP-\(\alpha\) mRNA and protein expression both at
steady state and following TLR signaling (Fig. 5B and data not shown).

We conclude that although immature-like DC obtained in response to IL-10 or SIRP-α ligation displayed a large array of coregulated genes, those two immature-like cells can be differentiated on the basis of unique gene expression profiles. The SIRP-α-stimulated DC appear different from IL-10-stimulated DC in that they selectively express a panel of neutrophils attracting chemokines, both at the mRNA and protein level. Finally, SIRP-α and IL-10 pathways seem to be mutually exclusive as IL-10 down-regulates SIRP-α expression whereas SIRP-α triggering down-regulates IL-10R expression in DC.

Discussion

DC maturation is often perceived as an irreversible process initiated by exposure to bacterial and/or inflammatory signals. At the close of this highly coordinated program, terminally differentiated DC are capable of presenting Ag in an immunogenic fashion to cognate T lymphocytes within secondary lymphoid organs. Whereas a large array of factors, collectively referred to as “danger signals,” can trigger DC maturation in APCs, only few were recognized as actively maintaining DC in an immature stage. IL-10 is a prototype inhibitor of DC maturation.

Here, we show that CD47/SIRP-α interaction and IL-10/IL-10R constituted two distinct and separate molecular pathways to alter DC response to danger signals. We have used throughout our study SAC and LPS as surrogates for danger signals from Gram-positive and Gram-negative bacteria and our unpublished preliminary data (M. Rubio and D. Braun) indicated similar results in response to poly-IC. The final common outcome of SIRP-α or IL-10 R ligation was the arrest of DC maturation and migration resulting in the down-regulation of the T cell response. Whereas IL-10 readily opposed DC maturation by preventing acquisition of mature DC phenotypic and functional features, the biological consequences of SIRP-α triggering concomitantly, or shortly after, the encounter with a danger signal only became apparent at a later phase of the maturation process. More precisely, upon SIRP-α triggering, DC could first acquire morphologic, phenotypic, and selected functional characteristics of mature DC but subsequently reverted to immature-like cells. We showed that this apparent reversion was accompanied by the reacquisition of immature DC functional attributes including high Ag uptake and cytokine production capabilities, low chemotaxis toward CCL19, and poor allostimulatory functions.

We thus propose that CD47/SIRP-α interaction is an active process that permits DC to become first “semimature” migratory cells in response to danger signals. Similar DC that promote tolerance are generated in vitro or in vivo with inflammatory mediators IL-1 and TNF-α (29, 36, 37). In fact, DC migration to the draining LN is not always correlated with full DC maturation in vivo in the steady state. In nonmanipulated healthy mice, iDC residing in peripheral tissues, continuously acquire self-Ags or cell-associated Ags (for instance: intestinal apoptotic epithelial cells, melanin granules, stomach parietal cells, or pancreas β cells), and transport them to the regional lymph nodes (LN) to induce tolerance (38–40). In humans, immature Langerhans cells accumulate in the LN of patients suffering from dermatopathic lymphadenopathy, further supporting the idea that maturation may not be strictly upstream of migration (41). As SIRP-α-ligated immature DC, IL-10-treated skin DC reportedly migrate efficiently toward CCL19 despite their quasi-immature state (42). Neither of these studies excluded that DC can migrate in a mature state and down-regulate costimulatory molecules once in the LN. A possibility raised by the kinetics presented here. Furthermore, two recent studies support the view that mature DC are not end cells (43). First, vitD3 redirects already differentiated DC toward a more immature phenotype (44). Second,
murine bone marrow-derived fully mature DC can further differentiate into regulatory DC under the influence of stromal cells (45).

The SIRP-α-mediated reversion into immature-like DC is completely IL-10 independent. First, IL-10 neutralization did not interfere with CD47 abortive effects. Second, endogenous IL-10 production was not increased but suppressed following CD47-Fc treatment. Third, both pathways appeared to reciprocally inhibit production was not increased but suppressed following CD47-Fc ligated DC did not acquire macrophage features, such as CD14 and FcγR (data not shown).

Finally, CD47-Fc and IL-10 each elicited a distinct profile of gene expression irrespective of the maturation and activation status of the DC as depicted in the “heatmap” (Fig. 4B). SIRP-α ligation did not provoke a general block in DC cytokine and chemokine production. Rather, it induced the secretion of CXCR2 ligands (i.e., neutrophil chemoattractants CXCL1, CXCL5, and CXCL6), CCL1, and IL-1 family members in response to danger signals. Thus, while it counteracts the ability of DCs to prime naive T cells, SIRP-α engagement on DC might simultaneously promote recruitment of neutrophils on the site. The latter ensure bacterial clearance; however, they may interact with DC to promote their maturation (46).

These data corroborate with the impaired neutrophil recruitment observed in CD47-deficient mice at the site of infection (47). Also, IL-10-pretreated murine bone marrow-derived DC produce, in response to TLR agonists, a novel soluble mediator binding to CXCR2, i.e., DC inflammatory protein-1, which may promote local inflammation (48).

We conclude that the CD47 and SIRP-α molecules may exert a unique role in the negative regulation of the immune response, distinct from that of IL-10. IL-10 is a major inducer of peripheral T cell tolerance. As such, IL-10 may be considered as a general counterregulator across the immune system (49). In contrast, SIRP-α is selectively expressed on APC and neutrophils. CD47 is present on hemopoietic and nonhemopoietic cells. Which cell type expressing CD47 precisely interacts with SIRP-α on DC and under which conditions does it happen in vivo? Schakel et al. (50) recently demonstrated that IL-12 p70 secretion is abundantly secreted in response to TLR ligands by a blood DC subset provided the contact between CD47 on erythrocytes and SIRP-α on DC is interrupted. Also, SIRP-α is engaged by CD47-expressing live but not apoptotic cells (25). Nonetheless, the difficulty in ascribing a precise cellular context for CD47/SIRP-α interaction stems from the fact that surface CD47 expression might be necessary but insufficient to warrant SIRP-α engagement. In situ, competition with alternative CD47 ligands (integrins, thrombospondin) might compromise CD47 availability. In addition, more subtle CD47 regulations such as conformational modulation linked to segregation in cell surface microdomains might limit engagement of SIRP-α by CD47. In agreement with this concept, Subramanian et al. (51) reported that CD47/SIRP-α interaction is cell-type specific.

Thus, we hypothesize that the CD47/SIRP-α interaction allows for maintenance of a basal state of DC activation centered on homeostatic function and propose that multiple cellular and molecular mechanisms could modulate CD47/SIRP-α interaction at the initiation and resolution phases of the immune response. The SIRP-α-induced final arrest of DC maturation combined to the proposed cell death of mDC in the LN would avoid the unnecessary activation of more naive T cells and thus lead to the arrest of the immune response.

Taken all, phenotypic maturity of a DC can no longer be seen as the sole criteria to appreciate its full potential to drive effector (protective vs tolerogenic) response. A two-step DC maturation program reportedly encompasses an initial licensing step mediated by danger signals followed by a full maturation step driven by CCL19 (52). We here propose that the semimaturation stage is a checkpoint controlled by CD47/SIRP-α interactions in the maturation process at which the DC may choose between completion of their maturation program and regression to functional immature-like cells. Thus, the manipulation of the SIRP-α-signaling pathway might be beneficial to the host because it dampens the inflammatory response without compromising the first line of defense against invading pathogens.

Acknowledgments

We thank Christian Charbonneau for assistance with confocal microscopy and Guy Delespesse, Lisa Walter, and Sylvie Lesage for discussions and critical reading of the manuscript.

Disclosures

The authors have no financial conflict of interest.

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15. Seiffert, M., P. Brossart, C. Cant, M. Cella, M. Colonna, W. Brugger, L. Kanz, and Guy Delespesse, Lisa Walter, and Sylvie Lesage for discussions and critical reading of the manuscript.

Disclosures

The authors have no financial conflict of interest.
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