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Francesca Granucci; ... et. al

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Early IL-2 Production by Mouse Dendritic Cells Is the Result of Microbial-Induced Priming[1]

Francesca Granucci,[2]* Sonia Feau,[2]*† Véronique Angeli,‡ François Trottein,‡ and Paola Ricciardi-Castagnoli[3]*

Dendritic cells (DCs) are professional APCs able to initiate innate and adaptive immune responses against invading pathogens. Different properties such as the efficient Ag processing machinery, the high levels of expression of costimulatory molecules and peptide-MHC complexes, and the production of cytokines contribute in making DCs potent stimulators of naive T cell responses. Recently we have observed that DCs are able to produce IL-2 following bacterial stimulation, and we have demonstrated that this particular cytokine is a key molecule conferring to early bacterial activated DCs unique T cell priming capacity. In the present study we show that many different microbial stimuli, but not inflammatory cytokines, are able to stimulate DCs to produce IL-2, indicating that DCs can distinguish a cytokine-mediated inflammatory process from the actual presence of an infection. The capacity to produce IL-2 following a microbial stimuli encounter is a feature shared by diverse DC subtypes in vivo, such as CD8α^+ and CD8α^- splenic DCs and epidermal Langerhans cells. When early activated DCs interact with T cells, IL-2 produced by DCs is enriched at the site of cell-cell contact, confirming the importance of DCs-derived IL-2 in T cell activation. ***The Journal of Immunology,*** **2003, 170: 5075–5081.**

The immune system is characterized by the ability to respond to infectious agents without mounting a destructive response against self-tissues. The first line of defense to invading pathogens is represented by the innate immune response that detects and limits the infection. Moreover, the innate response also contributes to the development of the adaptive immune response, which gives rise to immunological memory.

Among the cells that participate to innate responses, dendritic cells (DCs)[4] play a central role. They are extremely versatile APCs involved in the initiation of both innate and adaptive immunity (1), but also in the differentiation of regulatory T cells (2) required for the maintenance of self-tolerance.

How DCs can mediate these diverse and almost contradictory functions has been recently investigated. The plasticity of these cells allows them to undergo a complete genetic reprogramming in response to external microbial stimuli (3). Resting, immature DCs are highly phagocytic and continuously internalize soluble and particulate Ags that are processed and presented to T cells. The interaction of immature DCs with T cells induces an abortive T cell activation with the induction of T cell anergy (4, 5) or the differentiation of regulatory T cells (6). In contrast, microbial stimuli that are recognized through a complex DC innate receptor repertoire induce DC maturation that is completed after 24 h (3, 7). Mature DCs express at the cell surface high levels of stable peptide-MHC complexes and costimulatory molecules and efficiently prime naive T cells. During the process of differentiation, DCs undergo intermediate maturational stages in which they express, with a strictly defined kinetic, cytokines and cell surface molecules critical for the initiation and control of innate and, then, adaptive immune responses (3, 8). The extent and type of innate and adaptive responses induced by DCs are related to the type of signal they have received (7). Indeed, DCs are able to distinguish different pathogens through the expression of pattern-recognition receptors that interact with specific microorganism molecular structures called microbes-associated molecular patterns. These constitutive and conserved microbial structures are absent in host mammalian cells and represent the signature of microorganisms (9). Well-defined pattern-recognition receptors are Toll-like receptors (TLRs). The stimulation of different TLRs at the DC surface results in the activation of different signaling pathways and in the induction of diverse maturation processes that influence the outcome of adaptive immunity (7). In this sense DCs are able to respond in a pathogen-specific way.

We have, recently, used live bacteria to perturb immature DCs with the attempt to identify genes involved in DC genetic reprogramming. A global gene expression analysis of immature and activated DCs at different time points after bacterial encounter has been performed, and unexpectedly, the mRNA for IL-2 was found up-regulated at early time points after Gram-negative bacterial stimulation (3). In addition, IL-2 protein was produced and secreted by DCs essentially between 4 and 6 h after stimulation. This cytokine has been described as a NK, B, and T cell growth factor, and for this reason it may represent a key molecule conferring to DCs the unique ability to activate NK, B, and T cells. In agreement with this assumption, IL-2-deficient DCs were found severely impaired in their ability to prime CD4$^+$ and CD8$^+$ T cells in MLRs when compared with IL-2-sufficient DCs (3), and consistently, they were also inefficient in activating NK cell responses (F.

*Department of Biotechnology and Bioscience, University of Milano-Bicocca, Milan, Italy; †Institut National de la Santé et de la Recherche Médicale Unité 395, Claude de Preval Institute, Centre Hospitalier de l'Université Purpan, Toulouse, France; and ‡Center d'Immunologie et de Biologie Parasitaire, Institut National de la Santé et de la Recherche Médicale Unité 547, Institut Pasteur de Lille, Lille, France

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[2] F.G. and S.F. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Paola Ricciardi-Castagnoli, Department of Biotechnology and Bioscience, University of Milano-Bicocca, Piazza della Scienza 2, Milan 20126, Italy. E-mail address: paola.castagnoli@unimib.it

[4] Abbreviations used in this paper: DC, dendritic cell; LC, Langerhans cell; BMDC-sRAG2$^{-/-}$, dendritic cells generated in vitro from bone marrow of RAG2-deficient mice; LTA, lipoteichoic acid; PGN, peptidoglycan; TLR, Toll-like receptor; CsA, cyclosporin A; FLT3L, Flt3 ligand; MOI, multiplicity of infection.

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In the present study, we have analyzed the nature of the stimuli able to induce IL-2 production by DCs and investigated whether the tissue origin of DCs could influence this property. We show that only stimuli that are known to bind to TLRs, but not inflammatory cytokines, are able to induce IL-2 secretion by DCs and that IL-2 production is independent from the DC tissue origin. Indeed, immature DCs from spleen, bone marrow, or skin Langerhans cells (LC) secrete IL-2 following microbial stimuli activation. Moreover, when early activated DCs interact with T cells, IL-2 produced by DCs is enriched at the site of cell-cell contact, confirming the importance of DC-derived IL-2 in T cell activation. Finally, we confirm in vivo the capacity of DCs to produce IL-2 following bacterial, LPS, or zymosan injection.

Materials and Methods

Abs and reagents

LPS (*Escherichia coli* 026:B6, used at 10 μg/ml) and zymosan (used at 10 μg/ml) were obtained from Sigma-Aldrich (St. Louis, MO). rTNF-α (Genentech, South San Francisco, CA) and rIL-1β (Genzyme, Cambridge, MA) were used at 100 U/ml and 10 μg/ml, respectively. IFN-α was kindly provided by Schering-Plough (Dardilly, France) and used at 1000 U/ml. CpG (TCCATGACGTTCCTGATGCT) and CpG control (TCCATGA GCTTCCTGATGCT) oligos were purchased from Life Technologies (Rockville, MD) and used at a concentration of 1 μM. PE-conjugated anti-IL-2, PE-conjugated rat, isotype control, biotinylated anti-CD8α, and FITC-conjugated anti-CD11c mAbs were purchased from BD PharMingen (San Diego, CA). Quantum Red-conjugated streptavidin was obtained from Sigma-Aldrich. Anti-I-A^d/I-E^d mAb (clone M5/114, rat IgG2b) was kindly provided by Dr. A. Ager (National Institute of Medical Research, London, U.K.). FITC-conjugated anti-rat was from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-CD40 Ab (clone FGK45 (10)) was used at a concentration of 20 μg/ml and cross-linked with a monoclonal anti-rat Ab (10 μg/ml; BD PharMingen). Lipoteichoic acid (LTA) and peptidoglycan (PGN), from Sigma-Aldrich, were used at a concentration of 10 μg/ml.

Mice

Pathogen-free C57BL/6 and BALB/c mice were obtained from Harlan Breeders (Bresso, Italy) or from Iffa Credo (L'arbresle, France) for LC preparation and used at 6–10 wk of age. C57BL/6 and BALB/c RAG2$^{-/-}$ animals were obtained from Center de Distribution, de Typage et d'Archivage Animal (Orleans, France) and kept in pathogen-free conditions. All experiments were performed in compliance with relevant laws and institutional guidelines.

DCs and culture medium

D1 (mouse DC line derived from spleen) and D8 (mouse DC line derived from bone marrow) long-term DCs were cultured in IMDM (Sigma-Aldrich) containing 10% heat-inactivated FBS (Life Technologies), 100 IU penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Sigma-Aldrich), and 50 μM 2-ME (complete IMDM) with 30% supernatant from R1 medium (supernatant from NIH3T3 fibroblasts transfected with GM-CSF (11)). Fresh BMDCsRAG2$^{-/-}$ were derived from RAG2$^{-/-}$ BALB/c or C57BL/6 bone marrow cells. Usually bone marrow cells were cultured for 2 days and then frozen. BMDCsRAG2$^{-/-}$ were obtained from defrost cells after 7 days of culture in medium containing 10% of supernatant of GM-CSF-transduced B16 tumor cells (12). Only immature BMDCs-RAG2$^{-/-}$ were used for activation as judged by low B7.2 and CD40 expression. Cells showing a partial spontaneous activation were discarded. For IL-2 experiments 2×10^6 cells/well were plated in 6-well plates in 5 ml of medium.

IL-2 ELISA

IL-2 ELISA was performed using the DuoSet kit (R&D Systems, Minneapolis, MN) and following the manufacturer's recommendations.

In vivo analysis of IL-2 production by DCs

C57BL/6 mice were transplanted with B16 tumor cells transduced with Flt3 ligand (FLT3L) or GM-CSF (12). After 18 days mice were i.p. injected with 50 μg LPS, 10^8 *E. coli* (DH5α), or 500 μg zymosan. Three hours after treatment spleens were removed, and unicellular suspensions were made and incubated with brefeldin A (10 μg/ml; Sigma-Aldrich) for 1.5 h. Cells were fixed with 2% paraformaldehyde, permeabilized with PBS containing 5% FCS and 0.5% saponin, and stained with PE-labeled IL-2-specific and FITC-labeled CD11c-specific mAbs (BD PharMingen). Cells were then analyzed at the FACScan and fluorescence microscope.

Microbead phagocytosis

DCs were incubated for 3 h at 37°C with a cell:bead ratio of 1:100. Cells were then washed with PBS and incubated 5′ with 0.05% trypsin 0.53 mM of EDTA (Life Technologies) to eliminate any residual nonphagocytosed bead. The 1-μm PE-conjugated microbeads were from Molecular Probes (Eugene, OR). Cells were eventually fixed in paraformaldehyde 1% PBS to evaluate the uptake by FACScan (BD Biosciences, San Jose, CA) analysis. To evaluate IL-2 production, supernatants were tested by ELISA at the indicated time points.

Cell immunofluorescent labeling

Staining of DCs and T cells was performed as described (13). In brief, DCs were plated on coverslips and activated with LPS (10 μg/ml). CD4$^+$ T cells were purified from TCR OVA, DO11.10 BALB/c transgenic mice by negative selection of B220$^+$, CD8$^+$, Mac1$^+$, and CD11c$^+$ cells using Dynabeads (Dynal Biotech, Oslo, Norway) and added to DCs together with the OVA peptide (1 μg/ml) 1.5 h after LPS-activation. After 20 min incubation, cells were washed with PBS, fixed with 2% paraformaldehyde in PBS, and permeabilized with PBS containing 5% FCS and 0.5% saponin. Cells were then labeled with anti-IL-2 Ab for 30 min at room temperature.

Infection with bacteria and yeasts

E. coli DH5α were added to DCs at a multiplicity of infection (MOI) of 10. Cocultures were incubated for 1.5 h, then washed and supplemented with gentamicin and tetracycline at a final concentration of 50 μg/ml and 30 μg/ml, respectively. For activation with yeasts, DCs were pulsed with live *Saccharomyces cerevisiae* for 2 h before the addition of amphotericin B (Sigma-Aldrich) at a final concentration of 2.5 μg/ml.

Langerhans cells

Epidermal cells containing 1–3% LC were prepared from ear epidermis by standard trypsinization (14). Epidermal cells were incubated at 37°C in a 6-well tissue culture plate in 3 ml of culture medium (RPMI 1640, supplemented with 10% FCS serum, 200 mM L-glutamine, and 20 μg/ml gentamicin) (Life Technologies). After 12 h of incubation, epidermal cells were stimulated with LPS (10 μg/ml), and brefeldin A was immediately added. As negative control, cells were pretreated with cyclosporin A (CsA) 1 h before stimulation. After 6 h of stimulation, epidermal cells were double stained with anti-I-A^d/I-E^d mAb (clone M5/114, rat IgG2b) followed by FITC-conjugated anti-rat mAb. After surface immunostaining, cells were subjected to fixation with 2% paraformaldehyde and permeabilization with 0.1% saponin/1% BSA in PBS and then incubated with PE-conjugated anti-IL-2 mAb for intracellular IL-2 detection.

Results

IL-2 production by DCs is induced by microbial stimuli but not by inflammatory cytokines

DCs express a variety of functional TLRs that, once activated, transduce intracellular signals leading to the induction of genes, such as inflammatory cytokines, chemokines, and costimulatory molecules involved in the defense against invading pathogens. To investigate the stimuli that could induce IL-2 production, mouse DCs were stimulated in vitro with LPS, a component of Gram-negative bacteria that signals through TLR4 (15), oligo DNA containing the unmethylated CpG motif of bacterial DNA that is recognized by TLR9 (16), zymosan (yeast cell wall particles), LTA (a component of Gram-positive bacteria), and PGN from Gram-positive bacteria that are recognized by TLR2 (9, 17, 18). Two long-term growth factor-dependent (GM-CSF) mouse DC lines, one derived from spleen (D1) and one derived from bone marrow (D8), were used (11). To exclude the possibility that DC culture conditions (see *Materials and Methods*) could influence the results, DCs generated in vitro from bone marrow of RAG2-deficient mice (BMDCs/RAG2$^{-/-}$) differentiated in vitro in the presence of a source of GM-CSF different from the one used for long-term DC

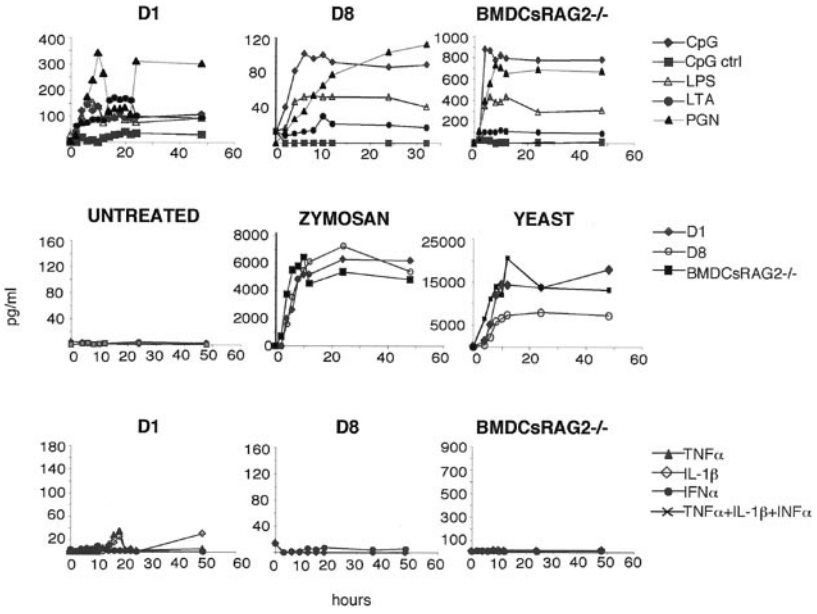

FIGURE 1. IL-2 production by DCs following yeasts, microbial cell products, and cytokine activation. Two long-term DC lines (D1 and D8 cells) and fresh short-term DCs derived from bone marrow of RAG2$^{-/-}$ mice (BMDCsRAG2$^{-/-}$) were stimulated with the indicated microbes, microbial cell products, and cytokines. IL-2 was measured by ELISA in the supernatant of activated cells at the indicated time points after stimulation. The entire experiment was repeated three times with similar results.

lines (see *Materials and Methods*) were also used. All of the microbial stimuli were able to induce DC phenotypic maturation (data not shown) and IL-2 production (Fig. 1). In general, a larger amount of IL-2 was produced by fresh BMDCs/RAG2$^{-/-}$ when compared with long-term DC lines, most likely because they have a lower threshold of activation. The only exception was zymosan that proved to be a very efficient stimulus in inducing IL-2 secretion by both long- and short-term DC lines. In agreement with this, the best stimulus for IL-2 production by DCs was represented by live yeasts (Fig. 1). CpG and PGN were also efficient in stimulating DCs to secrete IL-2.

Three different inflammatory cytokines, TNF-α, IL-1β, and IFN-α, were then tested for their ability to stimulate DCs to produce IL-2. TNF-α was selected because it is commonly used to activate immature DCs and to generate in vitro a large amount of DCs for cell-based cancer therapies (19). IL-1β has been used because its intracellular signaling pathway partially overlaps with the one of LPS (20), and IFN-α was used because it has been described as a good stimulus for DC activation (21). In contrast to what was observed for microbial stimuli, none of the inflammatory cytokines, used individually or added simultaneously to DC cultures, were able to promote IL-2 secretion by splenic or bone marrow-derived DCs, although they could induce DC phenotypic maturation (data not shown).

Thus, DCs produce IL-2 early after contact with a pathogen or its products regardless of their tissue origin, but not in response to specific inflammatory cytokines.

To verify whether the IL-2 produced by DCs was bioactive, we tested the capacity of bacterial-activated BMDCs/RAG2$^{-/-}$ supernatants to sustain IL-2-dependent CTLL growth. As shown in Fig. 2, CTLL were able to proliferate in the presence of bacterial-activated DC supernatants, and their growth was completely inhibited when the blocking anti-IL-2 Ab, S4B6, was added to the cultures.

Phagocytosis per se is not sufficient to induce IL-2 production by DCs

To investigate whether the phagocytic process per se could activate DCs in terms of IL-2 production, DCs were incubated with inert latex beads or Gram-negative bacteria (*E. coli* DH5α) as control, and IL-2 was measured in culture supernatant. As shown in Fig. 3*B*, inert latex beads alone, although efficiently phagocytosed (Fig. 3*A*), were not able to induce IL-2 production in both splenic or bone marrow DCs, confirming the previous observation that microbial stimuli are necessary for this process.

T cell-mediated stimuli induce IL-2 secretion by DCs

IL-2 secretion by DCs could also be induced by stimuli that mimic T cell signal, such as activation of CD40 by anti-CD40 Ab. Activation of CD40 was a stimulus as efficient as microbial stimuli in inducing IL-2 production by D1 cells (Fig. 4). In contrast, in BMDCsRAG2$^{-/-}$ cultures a little amount or no IL-2 could be detected in the supernatant at early time points following CD40 stimulation (Fig. 4). This could be because fresh immature BMDCsRAG2$^{-/-}$ express, at the cell surface, less CD40 (almost undetectable by cytofluorometric analysis) than D1 line. If DCs were first treated with LPS for 14 h and then subjected to CD40-activation, a second phase of IL-2 production could be induced (Fig. 4), indicating that early after activation with microbial stimuli, DCs are not refractory to further stimulation.

IL-2 production by DCs is not restricted to lymphoid tissues

To test whether DCs derived from nonlymphoid tissues could produce IL-2 upon stimulation, LC, prepared by trypsinization from murine epidermal sheets, were stimulated with LPS; 6 h later IL-2 production was analyzed by flow cytometry. As depicted in Fig. 5,

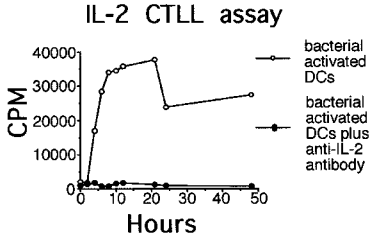

FIGURE 2. BMDCs/RAG2$^{-/-}$ produce bioactive IL-2. BMDCs/RAG2$^{-/-}$ were incubated with *E. coli* (MOI of 10); supernatant was collected at the indicated time points and tested for its IL-2 content on the CTLL cell line (5×10^3 cell/well in 96-well plates). CTLL proliferation was evaluated by [^{3}H]thymidine incorporation. The values represent mean cpm of duplicate wells.

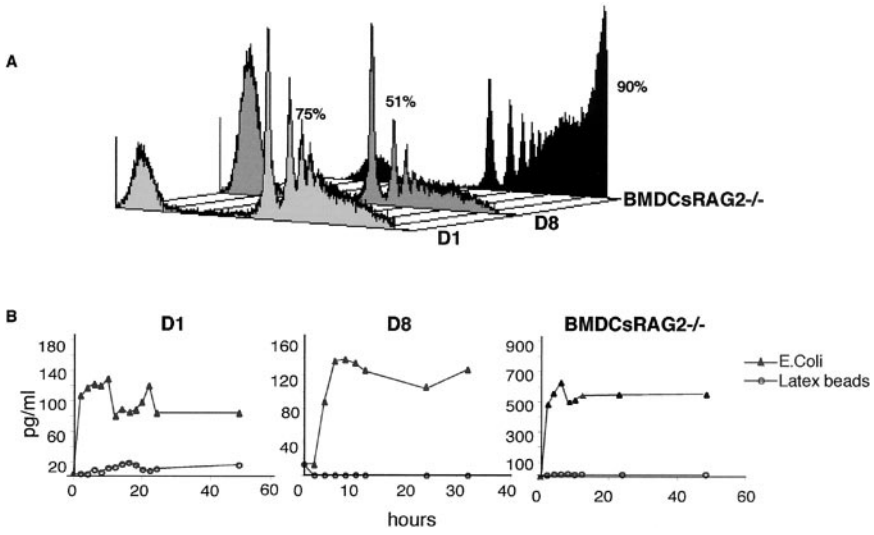

FIGURE 3. IL-2 production by DCs following incubation with Gram-negative bacteria or latex beads. D1 and D8 cell lines and BMDCsRAG2$^{-/-}$ were incubated with latex beads (100 beads per 1 cell) or *E. coli* (MOI of 10). *A*, The uptake efficiency was evaluated by FACS analysis. *B*, IL-2 in the supernatants was measured by ELISA at the indicated time points. The experiment was repeated twice with similar results.

a very limited number of unstimulated LC (MHC class II-positive cells) were IL-2 positive. This number strongly increased following LPS stimulation. To verify the specificity of IL-2 production, epidermal cells were treated with CsA before LPS stimulation, because CsA strongly reduces IL-2 production by DCs following LPS stimulation (our unpublished results). As shown, CsA treatment induces a marked reduction in the number of IL-2-producing LC compared with untreated cells.

DCs produce IL-2 following bacterial or LPS injection in vivo

We then investigated whether the ability to produce IL-2 was a general DC property shared also by DCs in vivo or a sole feature of in vitro differentiating cells. For this purpose, mice were transplanted with GM-CSF or FLT3L-transduced tumors to expand CD8α^-CD11c$^+$ or CD8α^+CD11c$^+$ splenic DC populations, respectively (22), and i.p. injected with LPS, *E. coli* DH5α, or zymosan to prime DCs. The presence of IL-2-expressing CD11c$^+$ DCs in spleens of LPS- or bacterial-treated mice was revealed by flow cytometry analysis. Three hours after bacterial, zymosan, or LPS treatments an IL-2-positive CD8α^-CD11c$^+$ DC population was apparent in GM-CSF-treated mice (Fig. 6*A*). The possibility that the IL-2-positive cells were in fact T lymphocytes that formed doublets with DCs was excluded by performing an immunocytochemistry analysis of splenic single-cell suspensions stained with anti-IL-2 and anti-CD11c Abs. IL-2-positive cells expressed CD11c, indicating that IL-2-producing cells were, indeed, DCs (Fig. 6*C*). The same analysis was performed on FLT3L-treated mice. After bacterial, zymosan, or LPS injection, CD8α^+CD11c$^+$ IL-2-positive cells could be detected (Fig. 6*B*). Immunocytochemistry analysis confirmed that IL-2-expressing cells were also CD11c-positive (Fig. 6*C*). The difference in the efficiency of soluble factors such as LPS, compared with particulate stimuli such as bacteria and zymosan, in inducing IL-2 expression by DCs in vivo has been consistently observed in all the experiments performed, and the difference may reflect the lower efficiency of particulate stimuli in reaching DCs in the spleen. Some IL-2-positive DCs were visible already 2 h after activation, although the peak was at 3 h and disappeared after 6 h in both GM-CSF and FLT3L-treated mice (data not shown).

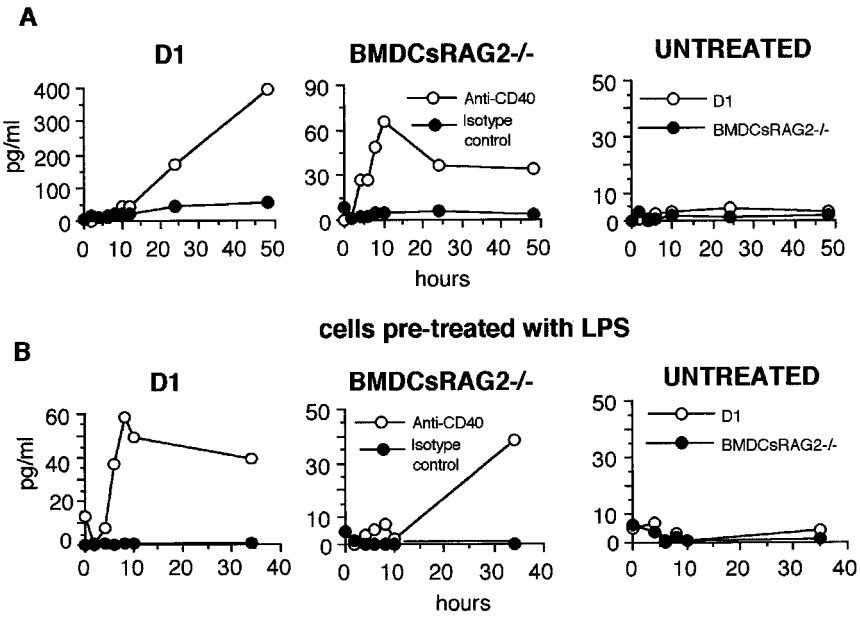

FIGURE 4. IL-2 production by DCs after CD40 stimulation. *A*, D1 cells and BMDCsRAG2$^{-/-}$ were activated with the anti-CD40 mAb, FGK45, or (*B*) preactivated with LPS for 14 h and then washed (time 0) before the incubation with the FGK45 Ab. At the indicated time points after stimulation, quantification of the amount of IL-2 present in the supernatants was made by ELISA.

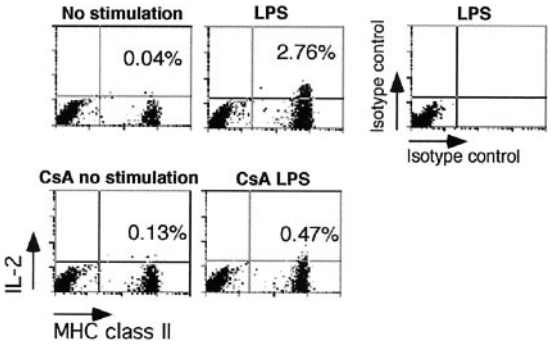

FIGURE 5. Production of IL-2 by LC after LPS stimulation. LC were stimulated with LPS in the presence or absence of CsA (1 μg/ml). Six hours after LPS stimulation, cells were double stained with anti-MHC class II and anti-IL-2 Abs. LC were identified as MHC class II positive. The experiment was repeated three times with similar results

In early activated DCs, IL-2 localizes at the interface between DC-T cell interaction

Naive T cell priming needs prolonged TCR stimulation that is achieved by the formation of an immunological synapse (23), a specialized molecular organization that takes place at the contact region between DC and T cell(s). The expression of high levels of costimulatory molecules and MHC-peptide complexes and the secretion of cytokines by DCs are at the origin of the high efficiency of DCs as APCs. As IL-2 is an additional molecule conferring DCs the unique naive T cell stimulatory capacity (3), it has been investigated whether IL-2 produced by early activated DCs is recruited at the contact region between interacting DC and T cells.

Immature or LPS-activated BMDCs/RAG2$^{-/-}$ were loaded or not with OVA-peptide, incubated with CD4$^+$ T cells from DO.11.10 transgenic animals for 20 min, and then stained with anti-IL-2 Ab. No IL-2 was produced by nonactivated DCs, and it was widely distributed in activated DC cytoplasm in the absence of T cells (Fig. 7, *A* and *B*). In the presence of OVA peptide, IL-2 produced by activated DCs was enriched at the interface between DCs and T cells (Fig. 7, *D* and *E*) in most of the cases analyzed, confirming the relevance of DC-derived IL-2 in the process of T cell activation. IL-2 enrichment at the site of contact was less frequent in the absence of peptide (Fig. 7, *C* and *E*).

Discussion

A unique feature of DCs is their ability to activate NK, B, and T cell responses. Whereas the mechanisms responsible for NK and B cell activation by DCs are mostly unknown, activation of T cells by DCs depends on many factors, such as the efficiency of processing machinery, the high levels of expression of costimulatory

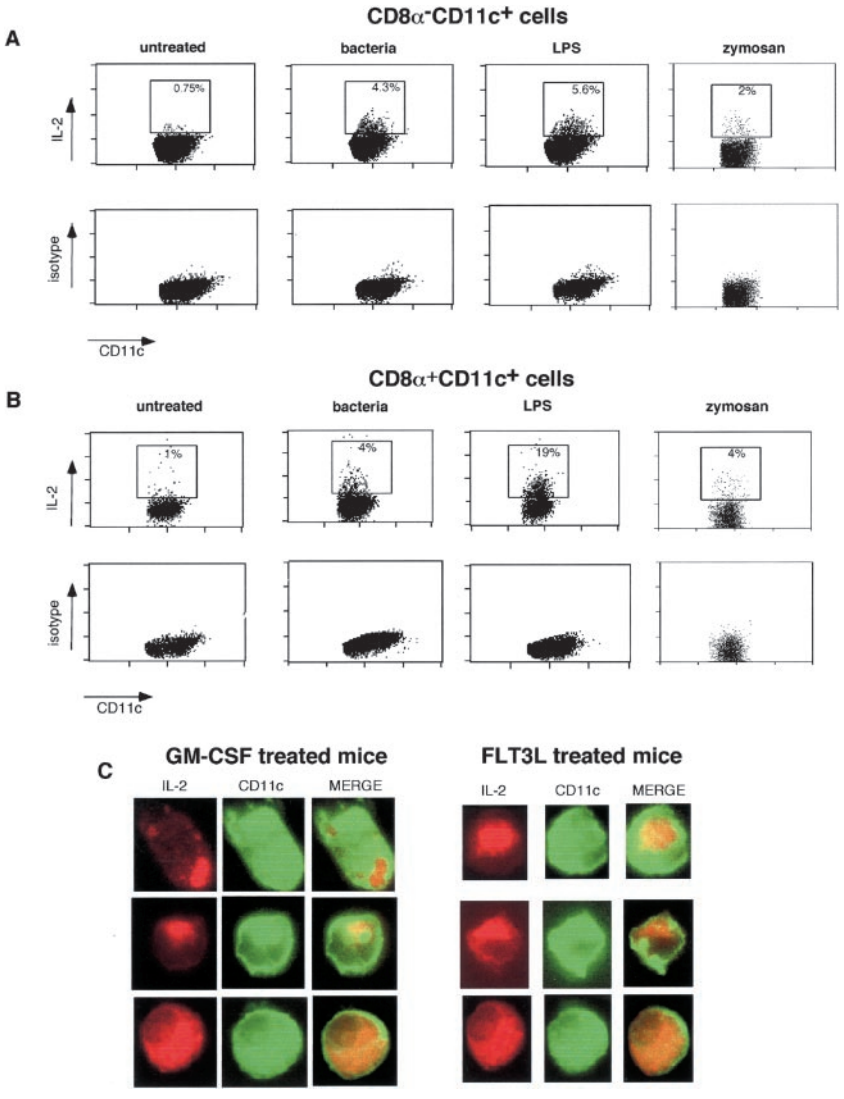

FIGURE 6. IL-2 production by DC subtypes in vivo. Mice were transplanted with GM-CSF (*A*) or FLT3L (*B*) transduced B16 tumor cells to expand, respectively, splenic CD8α^- and CD8α^+CD11c$^+$ DCs. Two weeks later, mice were injected i.p. with 10^8 bacteria (*E. coli*, DH5α) or LPS (50 μg) or zymosan (500 μg). Triple staining with anti-CD8, anti-CD11c, and anti-IL-2 or isotype control Abs was performed 3 h after bacterial, LPS, or zymosan treatments. *A*, Plots were gated on CD8α^-CD11c$^+$ cells. *B*, Plots were gated on CD8α^+CD11c$^+$ cells. *C*, Immunofluorescence images of splenic DCs from mice treated with FLT3L or GM-CSF. In the total splenic population, IL-2 positive cells (red) were examined for the expression of CD11c (green). The images are representative of many different double-positive cells that have been identified in the samples.

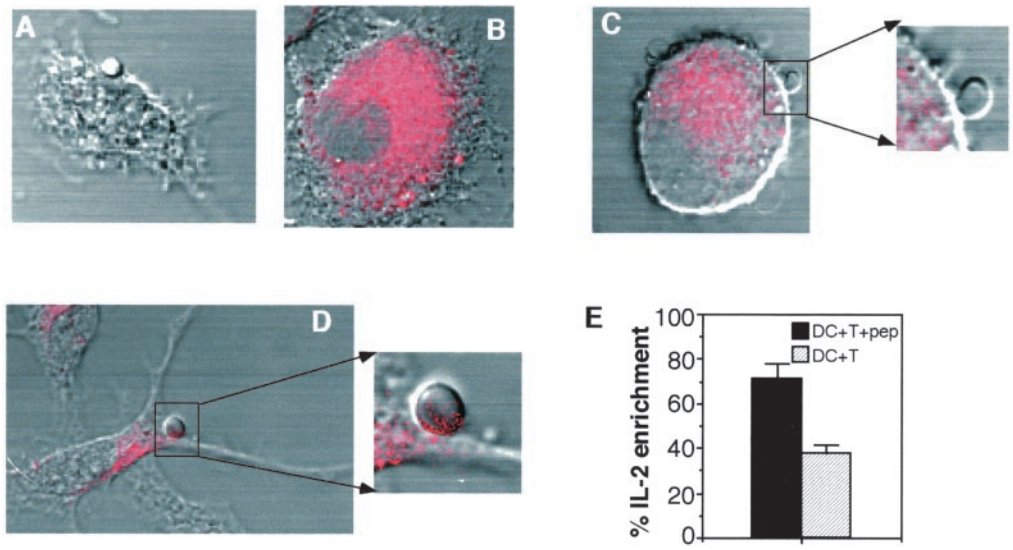

FIGURE 7. IL-2 produced by early activated DCs is enriched at the site of contact with T cells. Immature or 1.5 h LPS-activated BMDCsRAG2$^{-/-}$ were incubated with TCR OVA transgenic T cells for 20 min in the presence or absence of OVA peptide. Control experiments were also performed using activated DCs not incubated with T cells. Cells were then stained with anti-IL-2 (red) Ab. Differential interference contrast images of a DC or a T cell contacting a DC have been superimposed with fluorescence images and analyzed with a confocal microscope. *A*, Nonactivated DCs in the presence of T cells; *B*, activated DCs in the absence of T cells; *C*, activated DCs in the presence of T cells without the OVA peptide; *D*, activated DCs pulsed with the OVA peptide and cultured in the presence of T cells. *E*, Percentage of activated DC T cell conjugates exhibiting IL-2 enrichment at the contact sites in the presence (DCs + T + pep) or absence (DCs + T) of OVA peptide were counted in randomly selected fields. Results represent mean and SD calculated from three independent experiments.

molecules and peptide-MHC complexes, and the production of polarizing cytokines (24). Recently, our demonstration that DCs produce IL-2 early after bacterial stimulation (3) has opened new possibilities in understanding interactions of DCs with T cells and, probably, also with NK and B cells. Thus, it was important to define the nature of the stimuli that induce IL-2 secretion by DCs in vitro and the ability of DCs to produce IL-2 in vivo.

DCs were able to secrete IL-2 following activation with microbial cell products that signal through different TLRs. The early production of IL-2 may be involved in sustaining both innate NK and adaptive T cell responses. In contrast, none of the inflammatory cytokines examined induced IL-2 secretion by DCs, indicating that these cells can distinguish between a cytokine-mediated inflammatory process and the actual presence of an infection. Therefore, IL-2 production by DCs can presumably occur only during the early phases of an infection, when microorganisms or microbial cell products are in fact present, and not during the late phases of the inflammatory response when microbes have been eliminated and inflammation is sustained by cytokines. Although IL-1R, TLR4, TLR2, and TLR9 share several signaling components (9), DCs were not able to secrete IL-2 even after IL-1β interaction. The fact that cytokines were not capable of stimulating IL-2 production by DCs is in accordance with the evidence that the quality of the stimulus influences the DC maturation process and that inflammatory cytokines are not able to drive DCs to a level of maturation suitable for efficient priming of T cell responses (25). Indeed, it has been shown that DCs matured in the presence of TNF-α are rather tolerogenic (26).

Yeasts and zymosan, particles of yeast cell wall, were the most efficient stimuli for the induction of IL-2 secretion by DCs. The massive production of IL-2 by DCs following yeast and zymosan encounter could explain why yeasts act as a potent adjuvant, augmenting the ability of DCs to prime CD4$^+$ and CD8$^+$ T cells and to induce protective antitumor immunity upon adoptive transfer in vivo (27).

DC-derived IL-2 could be required to efficiently prime innate NK (F. Granucci, I. Zanoni, N. Pavelka, and P. Ricciardi-Castagnoli, manuscript in preparation) and acquired T cell responses (3) to resolve infections. The role of DC-derived IL-2 in T cell priming is supported by the fact that immunosuppressive viruses, such as CMV, that establish persistent infections block IL-2 production by activated DCs and affect the capacity of DCs to activate T cells (28). Among APCs, only DCs and not macrophages are able to produce IL-2 following bacterial encounter (3). Exogenous sources of IL-2 may be important for effective T cell priming when the frequency of Ag-specific T cells or their TCR affinity for peptide-MHC complexes is low. This could frequently happen in vivo during immune responses to microorganisms. Indeed, T cells able to mount a specific response are rare and carry different TCRs with heterogeneous affinities. Moreover, as DCs display at the surface thousands of different peptides derived from the processing of entire microorganisms, the amount of a particular peptide-MHC complex that can be recognized by a given T cell may be extremely reduced. Thus, in this context, exogenous IL-2 may represent an important costimulatory molecule to help T cell proliferation by activating and maintaining the expression of IL-2Rα chain.

It is commonly believed that DCs acquire the ability to prime naive T cell responses late after activation when they express maximal levels of peptide-MHC complexes and costimulatory molecules. Nevertheless, the early kinetic of IL-2 production could make DCs able to prime T cells almost immediately after microorganism encounter (3). This is in agreement with the observation that in vivo the first signs of naive CD4$^+$ T cell priming by DCs can be detected within 1 or 2 h following Ag administration (29).

Another role of IL-2 produced by DCs could be the homeostatic maintenance of regulatory T cells that express high levels of high affinity IL-2R and proliferate in response to IL-2 but are not able

to produce it (30). In fact, it is quite clear that IL-2 and costimulatory molecules play an important role in the homeostasis of regulatory T cells as this population is strongly reduced in IL-$2^{-/-}$, $CD28^{-/-}$, $CD40^{-/-}$, and $B7^{-/-}$ mice (31). Thus, in the gastrointestinal tract $CD4^+CD25^+$ regulatory T cells could survive by interacting with IL-2-producing and costimulatory molecules expressing DCs that have been recently activated by LPS or LTA derived from the commensal flora.

It has been described that DCs acquire the ability to directly prime $CD8^+$ T cell responses after encountering Ag-specific $CD4^+$ T cells (32–34). The mechanism proposed to explain this phenomenon is the activation of DCs through CD40-CD40L (32–34). IL-2 produced by DCs after CD40 activation could be the key molecule that helps $CD8^+$ T cell priming. This may be true for both nonactivated and LPS-matured DCs, because CD40-stimulation induced IL-2 production by immature DCs and also a second late phase of IL-2 secretion by LPS-activated DCs. Interestingly, when early activated DCs interact with T cells, IL-2 is recruited at the site of contact, suggesting that secretion of IL-2 by DCs is relevant for T cell activation. It is not surprising that also in the absence of peptide, IL-2 is enriched at the site of contact between early activated DCs and T cells in a high percentage of cases, because immunological synapses can form between T cells and DCs even in the absence of Ag (13). Different subsets of DCs were able to produce IL-2. Tissue resident LC as well as $CD8\alpha^+$ and $CD8\alpha^-CD11c^+$ splenic DCs became IL-2 positive following LPS-stimulation. $CD8\alpha^+$ and $CD8\alpha^-CD11c^+$ DCs are equally efficient in activating T cell responses, and consistently, both populations are able to produce IL-2 following LPS, zymosan, or bacterial activation.

IL-2 production by LC could be important for early NK cell activation in peripheral tissues. This process may be relevant to promote macrophage activation and to sustain inflammation, because activated NK cells produce large amount of IFN-γ.

Taken together, these observations suggest a molecular mechanism to explain the DC central role in priming both early and late immune responses. DC-derived IL-2 could be a key factor regulating and linking innate and adaptive immunity.

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