Brief Definitive Report

Ligation of CD40 on Dendritic Cells Triggers Production of High Levels of Interleukin-12 and Enhances T Cell Stimulatory Capacity: T-T Help via APC Activation

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Summary

We investigated the possibility that T helper cells might enhance the stimulatory function of dendritic cells (DCs). We found that ligation of CD40 by CD40L triggers the production of extremely high levels of bioactive IL-12. Other stimuli such as microbial agents, TNF-α or LPS are much less effective or not at all. In addition, CD40L is the most potent stimulus in upregulating the expression of ICAM-1, CD80, and CD86 molecules on DCs. These effects of CD40 ligation result in an increased capacity of DCs to trigger proliferative responses and IFN-γ production by T cells. These findings reveal a new role for CD40-CD40L interaction in regulating DC function and are relevant to design therapeutic strategies using cultured DCs.

It is well established that IL-12 is responsible for the development of Th1 responses (1–3). The identification of the cells responsible for IL-12 production in vivo at the site of T cell priming is a critical issue that needs to be clarified. In a series of original experiments Murphy, O'Garra, and colleagues demonstrated that bacteria such as *Listeria monocytogenes* can stimulate macrophages to produce IL-12 and that this cytokine can act on bystander T cells that recognize antigen on different APCs (1). Thus, in this system IL-12 appears to function in a paracrine fashion, since IL-12 production and antigen presentation could be carried out by different cells.

Dendritic cells (DCs) are professional APCs specialized in antigen capture, migration to secondary lymphoid organs and T cell priming (4–7). In particular, DCs can be triggered by proinflammatory stimuli such as TNF-α, IL-1, and LPS to mature and to upregulate adhesion and costimulatory molecules (7, 8). Recent reports indicate that DCs can produce IL-12, but the signals involved in IL-12 stimulation have not been fully characterized (9, 10).

We have tested IL-12 production by human DCs in response to various stimuli. CD40L appears to be the most if not the only effective stimulus since it induces the production of massive amounts of bioactive IL-12 in both primary and cultured DCs. In addition, it induces a marked upregulation of adhesion and costimulatory molecules, resulting in enhanced T cell stimulatory capacity. These results suggest that T cells can modulate by cognate interaction the antigen-presenting function of DCs and favor the development of a Th1 response.

Materials and Methods

Media and Reagents. The medium used throughout was RPMI 1640 supplemented with 2 mM L-glutamine, 1% non essential amino acids, 1% pyruvate, 50 μg/ml kanamycin (GIBCO BRL, Gaithersburg, MD), 5×10^{-5} M 2-ME (Merck, Darmstadt, Germany), and 10% FCS (Hyclone Laboratories, Logan, Utah). Human rIL-4 and rGM-CSF were produced in our laboratory by PCR cloning and expression in the myeloma expression system (11). Human rTNF-α was a gift of Dr. W. Lesslauer (Hoffmann-La Roche AG, Basel, Switzerland) and was used at a concentration of 20 ng/ml. LPS (*Salmonella abortus-equi*) was purchased from SEBAK (Munich, Germany). Growth curves of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* were established and cultures stopped in the logarithmic growth phase, washed twice in PBS and inactivated by incubating for 60 min at 60°C. They were used within a range of concentrations found to be optimal for stimulation of IL-12 production by mouse macrophages. J558L cells transfected with CD40L were previously described (12). The generation of Tetanus Toxoid-specific T cell clones was previously reported (13).

Primary and Cultured DCs. The method for the generation of DCs from peripheral blood monocytes has been previously described (14). Briefly, PBMCs obtained by Ficoll-Paque method (Organon Teknika, Durham, NC) were either separated on multistep Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) or isolated by elutrial centrifugation. The recovered

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monocytes, that were >90% pure as shown by flow cytometry with anti-CD14 antibody TIB228 (IgG2b; American Type Culture Collection, Rockville, MD), were cultured at 3×10^5/ml in RPMI-10% FCS supplemented with 50 ng/ml GM-CSF and 1,000 U/ml IL-4 for 6 days. In some experiments, primary DCs were isolated by serial depletion of T cells, monocytes, NK and B cells, as described (15). The purified cells were cultured for 24 h in RPMI-10% FCS supplemented with either GM-CSF+IL-4, GM-CSF+TNF-α, or GM-CSF alone. To induce IL-12 production, 1.5×10^5 cells were cultured in 0.5 ml RPMI-10% FCS in a 48 well plate in the presence of various stimuli. The supernatant was collected after 40 h.

Staining with mAbs. Cell staining was performed using mouse mAbs followed by FITC-conjugated, affinity-purified, goat anti-mouse IgG antibody (Southern Biotechnology, Birmingham, AL). The following mAbs were used: SPLV3 (IgG2a, anti-DQ) (American Type Culture Collection); B7.24 (IgG2a, anti-CD80) (kindly provided by Dr. M. Deboer, Immunogenetics, Ghent, Belgium); B70/B7-2 (IgG2b, anti-CD86) and HA58 (IgG1, anti-ICAM1) (Pharmingen, San Diego, CA). The samples were analyzed on a FACScan® (Becton Dickinson) using propidium iodide to exclude dead cells.

Staining for Intracellular IL-12. DCs from GM-CSF+IL-4 cultures were cultured with CD40L-transfected cells for 14 h, fixed in 0.1% of glutaraldehyde and 3.7% of formaldehyde, permeabilized in 0.2% of Triton X-100 and stained with the anti-IL-12 p40 mAb 2-4A1 (IgG2b) followed by FITC-conjugated second antibody and counter-stained with BODIPY TR-X phallacidin (Molecular Probes, Leiden, Holland). The cells were examined using a confocal microscope (MRC 1024) (Bio-Rad, Cambridge, MA).

Mixed Lymphocyte Reaction. 10^5 responding cells either from an unrelated individual (allogeneic MLR) or autologous PBMCs (autologous MLR) were cultured in 96-well flat-bottom microplates (Costar Corp.) with different numbers of irradiated (3,000 rad from a ^{137}Cs source) stimulator DCs cells in the presence or absence of anti-IL12 antibodies 20C2 (anti-p75, IgG1) and 2-4A1 at 2 μg/ml. Thymidine incorporation was measured on day 5 by a 18-h pulse with [^{3}H]thymidine (1 μCi/well, specific activity, 5 Ci/mMol; Amersham Life Science, Buckinghamshire, UK). Before the addition of [^{3}H]thymidine 50 μl of supernatants were collected to measure γ-IFN production.

Detection of IL-12 and IFN-γ by ELISA. IFN-γ was detected in the supernatants using a two-site sandwich ELISA (16); IL-12 p75 heterodimer and IL-12 p40 were measured by a two-site sandwich ELISA employing clone 20C2 and clone 2-4A1 as capture antibody, respectively; clone 4D6-conjugated to horse radish peroxidase (HRP) was used as second antibody (anti-IL12 antibodies were kindly provided by Dr. M. Gately, Hoffmann-La Roche, Inc., Nutley, NJ) (17). Samples were analyzed in serial twofold dilutions in triplicate; the sensitivity of the assay is 50 pg/ml.

Results

CD40 Ligation Is the Most Effective Stimulus Inducing IL-12 Production by DCs. We have previously shown that DCs can be generated after in vitro culture of peripheral blood mononuclear cells with GM-CSF+IL-4 (14). These cells are characterized by a very high endocytic activity and can respond to inflammatory stimuli such as TNF-α, IL-1 and LPS by undergoing a terminal maturation which results in downregulation of endocytic capacity and upregulation of adhesion and costimulatory molecules (7). Since IL-12 plays an important role in polarizing T cells towards Th1, it was important to ask whether DCs can produce IL-12 and to identify the most effective stimuli in triggering its production.

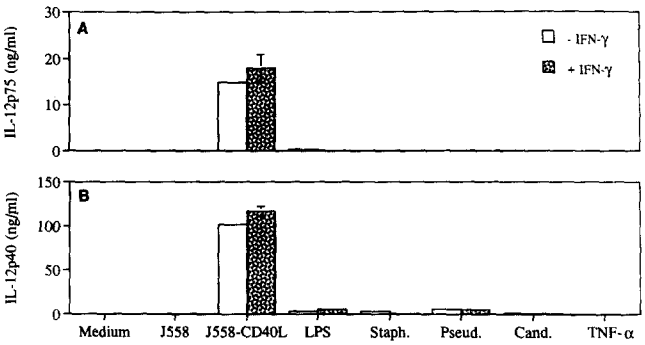

Figure 1. Cultured DCs produce high levels of bioactive IL-12 following stimulation by CD40L. DCs cultured in GM-CSF+IL4 were exposed to the indicated stimuli in the presence (*dotted bars*) or absence (*empty bars*) of IFN-γ. IL-12 p75 (*A*) and p40 (*B*) were measured after 40 h.

DCs generated in the presence of GM-CSF+IL-4 were challenged with various stimuli consisting of intact pathogens, LPS, TNF-α, and CD40L-transfected cells. IL-12 production was measured in the supernatants after 40 h using specific ELISA assays that detect either IL-12 p40 or the biologically active heterodimer p40/p35 (IL-12 p75). As shown in Fig. 1, DCs produced massive amounts of both p40 (up to 120 ng/ml) and p75 (up to 20 ng/ml) when stimulated by CD40L. The addition of IFN-γ enhanced IL-12 production in most preparations, but only to a limited extent. Other stimuli that have been reported to induce IL-12 production by murine macrophages were either much less effective or ineffective at all. These include LPS, TNF-α, and pathogens such as *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans*, and *Leishmania major.*

IL-12 production could be visualized at a single cell level by staining DCs, stimulated for 14 h with CD40L-transfected cells (Fig. 2). In fixed and permeabilized cells the IL-12 staining was typically localized in the Golgi apparatus, and was not detected in DCs cultured with control J558L cells (data not shown).

IL-12 production could be triggered also in an antigen-specific system. Fig. 3 shows that a tetanus toxoid-specific T cell clone (TC), in the presence of the relevant antigen, can trigger IL-12 production by antigen-presenting DCs.

To analyze whether IL-12 production is a general property of DCs we tested primary DCs isolated by serial depletion of T, monocytes, B and NK cells (15). As shown in Fig. 4, CD40L stimulation (but not LPS) induced production of extremely high levels of bioactive IL-12. It is noteworthy that such a high IL-12 production was observed also in cells that had not been cultured with IL-4, ruling out the possibility that the IL-12 production observed in these experiments could be due to the effect of IL-4 in the culture medium (18). In contrast to DCs, fresh monocytes produced very low levels of IL-12 (<1 ng/ml of IL-12

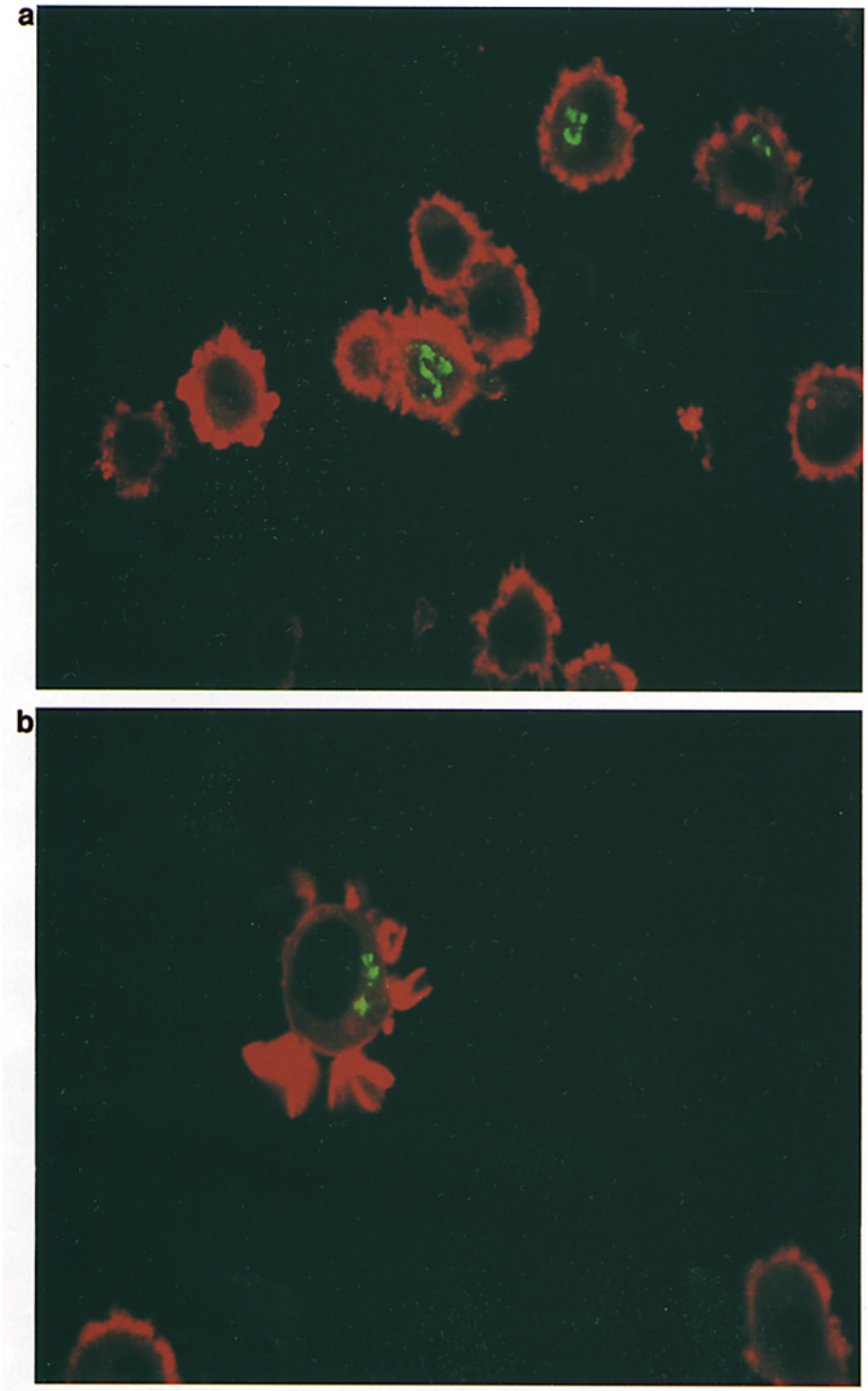

Figure 2. Induction of IL-12 p40 detected at single cell level in DCs stimulated with CD40L. DCs were stimulated with CD40L-transfected J558L cells and stained after 14 h with the anti IL-12 p40 mAb 2-4A1, followed by FITC-labeled second antibody and counter-stained with BODIPY TR-X phallacidin. More than 80% of DCs were positive.

p75), only when triggered by CD40L and, only in the presence of IFN-γ (data not shown).

We conclude that both primary and cultured DCs can respond selectively to stimulation by CD40L with production of extremely high levels of IL-12.

CD40 Ligation on DCs Results in Upregulation of Adhesion and Costimulatory Molecules and in Enhanced T Cell Stimulatory Capacity. We have previously shown that DCs cultured in GM-CSF+IL-4 have an immature phenotype and can undergo phenotypic and functional changes after stimulation with TNF-α, LPS, or CD40L (7). These stimuli may be actually responsible for DCs maturation in vivo and can be acting at different times during maturation in vivo. It was therefore interesting to investigate whether such stimuli were equally effective in modulating DCs surface antigens and in triggering T cell stimulatory properties.

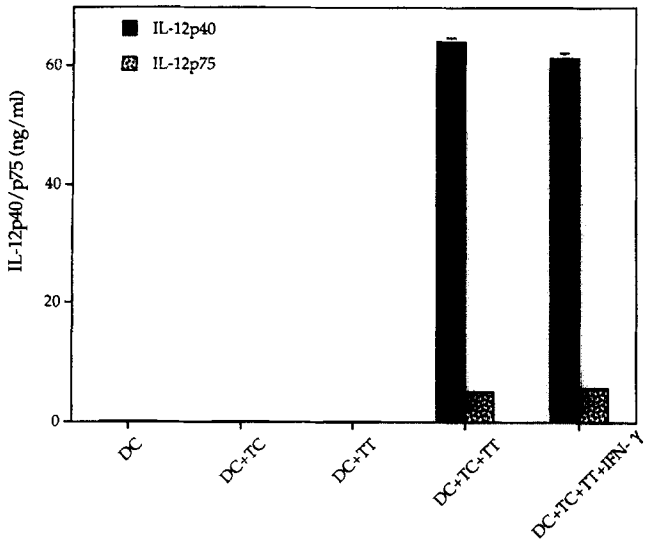

Figure 3. IL-12 production by DCs is elicited by antigen-specific interaction with T cells. DCs were cultured with a TT-specific T cell clone (*TC*) in the presence or absence of TT. IL-12 p40 (*black bars*) and p75 (*dotted bars*) were measured in the culture supernatants after 48 h. We exclude the T cell clone as a source of IL-12, since it did not produce IL-12 when stimulated by peptide presented by fixed EBV-B cells or by PMA + ionomycin.

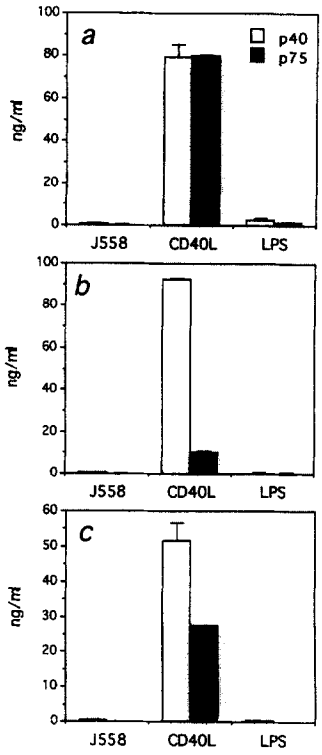

Figure 4. IL-12 production by primary DCs stimulated with CD40L. Primary DCs were isolated and cultured for 24 h with GM-CSF+IL-4 (*a*), GM-CSF+TNF-α (*b*) or GM-CSF alone (*c*). The cells were stimulated for 40 h with LPS or CD40L-J558L. IL-12 p40 (*empty bars*) and p75 (*filled bars*) production was measured.

We thus measured the level of adhesion and costimulatory molecules on DCs before and after stimulation. Table 1 shows that CD40L can dramatically increase the expression of ICAM-1 (up to six-fold the basal level), CD80 (~10-fold) and especially CD86 (up to 100-fold). In addition, the expression of HLA-DQ was increased ~10-fold. In all cases TNF-α and LPS had similar effects, although less pronounced.

DCs from GM-CSF+IL-4 cultures were stimulated with CD40L, TNF-α or LPS and compared for their stimulatory capacity in autologous and allogeneic MLR. The results in Fig. 5 show that in all cases the stimulatory capacity of DCs is dramatically enhanced by these maturation-inducing stimuli, the most effective stimulus being, however, CD40L. This was particularly evident when the production of IFN-γ was measured. Consistent with the role of IL-12 in driving Th1 responses (19), the addition of anti-IL-12 antibodies dramatically decreased IFN-γ production.

We conclude that CD40 ligation can increase the T cell stimulatory capacity of DCs and favor Th1 responses.

Discussion

We have shown that DCs can respond to stimulation via CD40L by producing very high levels of bioactive IL-12 and upregulating adhesion and costimulatory molecules. Altogether these effects lead to a stronger T cell stimulation and bias the T cell response towards Th1.

The level of IL-12 produced by cultured as well as primary DCs is much higher than that previously reported for macrophages and monocytes. The fact that DCs are likely to deliver IL-12 in a cognate fashion to T cells clustered around them makes this source of IL-12 even more effective for T cell stimulation.

Delespesse and coworkers have recently demonstrated that monocytes cultured for 48 h in GM-CSF and IFN-γ produce low levels of IL-12 (50–100 pg/ml IL-12 p75) after stimulation via CD40L (20). It is not clear how these cells relate to fresh monocytes or DCs. In our experiments fresh monocytes produce small amounts of IL-12 p75 and only when triggered by CD40L and in the presence of IFN-γ.

It is interesting that DCs appear to be very selective in their capacity to produce IL-12 in response to other stimuli, since they respond only to CD40L. Indeed other stimuli such as LPS and TNF-α, which share with CD40L the capacity to trigger DCs maturation, are less efficient or ineffective in driving IL-12 production by DCs. In addition,

Table 1. *DCs Upregulate MHC Class II, Adhesion and Costimulatory Molecules after Stimulation with CD40L. TNF-α, or LPS*

	DQ	ICAM-1	CD80	CD86
unstimulated	293	756	125	20
TNF-α	2060	1595	258	363
LPS	1634	1726	677	1605
CD40L	3461	5395	1100	2280

DCs from GM-CSF+IL-4–dependent cultures were stimulated as in Fig. 5 and analyzed after 48 h. The data show the median fluorescence intensity. The background (15–30) was subtracted. Comparable results were obtained in five separate experiments.

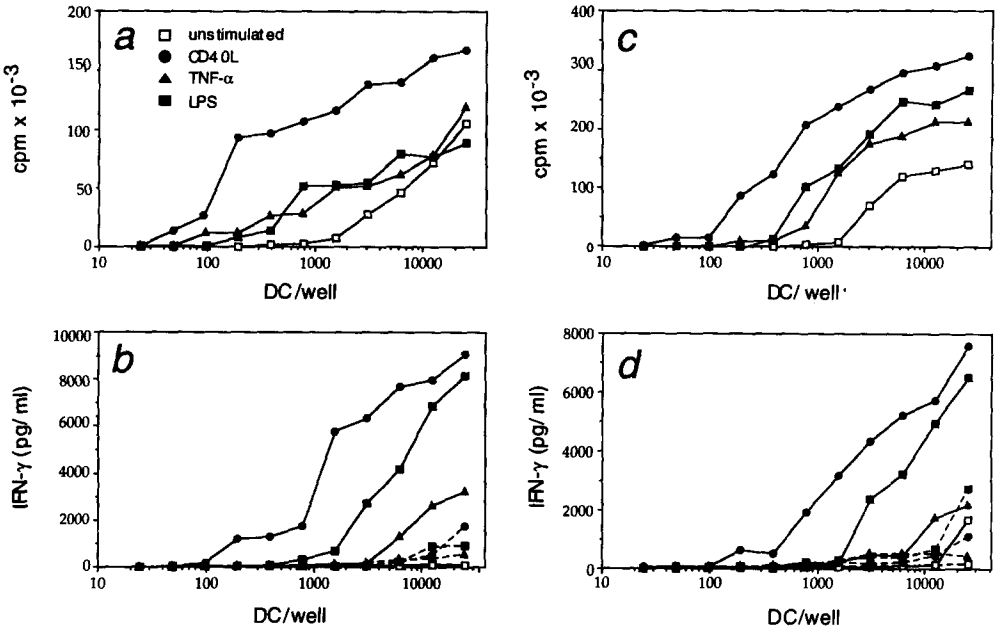

Figure 5. The capacity of DCs to stimulate T cell proliferation and IFN-γ production in autologous and allogeneic MLR is controlled by maturation-inducing stimuli. DCs from GM-CSF+IL-4 dependent cultures were stimulated for 40 h with LPS (■), TNF-α (▲) or irradiated CD40L-J558L (●), or untreated (□) and used as stimulators in autologous MLR (*a* and *b*) or allogeneic MLR (*c* and *d*). T cell proliferation (*a* and *c*) and IFN-γ production (*b* and *d*) were measured on day 5. In some cultures (*dotted line*), anti IL-12 antibodies were added. DCs cultured with control J558L cells gave results comparable to those of untreated DCs.

DCs do not produce detectable levels of IL-12 p75 in response to bacteria, which represent a very effective stimulus for macrophages.

Our results indicate that the CD40-CD40L interaction is essential for triggering IL-12 production by DCs, which may be the critical mechanism for induction of Th1 responses in vivo. Accordingly, the lack of protective T cell responses in CD40L-deficient mice (21) could well be explained by the inability of T cells to trigger IL-12 production by DCs with consequent failure to generate an efficient Th1 response.

On the basis of these data the following scenario could be proposed: (*a*) immature DCs scattered in peripheral tissues respond to pathogens or to TNF-α, produced as a consequence of tissue damage, by maturing and migrating to the T cell areas of the draining lymph nodes where they efficiently present antigen to T cells; (*b*) activated/memory T cells, that recognize antigen on DCs, readily express CD40L and efficiently stimulate IL-12 production by DCs as well as further upregulate DCs adhesion and costimulatory capacity. Since many T cells are known to cluster around a single DC, the induction of IL-12 production and the upregulation of the stimulatory capacity will result in an increased stimulation for many different T cells grouped around the same DC. This will result in T-T help via APC activation.

In this sequence of events the activation of APCs requires the interaction of DCs with primed T cells capable of expressing CD40L. These may be present as a consequence of previous immunizations. Alternatively, DCs stimulation may be induced by T cells specific for non peptidic ligand like some γδ+ (22) or α/β+ $CD4^-$ $CD8^-$ T cells (23).

Our results suggest that immunization with a T helper epitope might facilitate priming of CTLs by targeting CD40L+ Th1 cells on APCs. This model may explain old and new findings in which the generation of specific CTLs is enhanced by the upregulation of costimulatory signals such as B7 (24).

Finally, our results are relevant to the therapeutic use of cultured DCs as adjuvants for immunization. The inclusion of a universal T helper epitope (25) may target memory Th cells to DCs in vivo thus enhancing their capacity to present and trigger T cells specific for any antigen that is administered simultaneously on the same cells.

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