Critical Impact of the Kinetics of Dendritic Cells Activation on the in Vivo Induction of Tumor-specific T Lymphocytes

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ABSTRACT

Dendritic cells (DCs) need activation for the priming of antigen-specific immune responses. Recently activated DCs were described to prime in vitro strong T helper cell type 1 (Th1) responses, whereas at later time points, the same cells preferentially prime Th2 cells [Langenkamp, A. et al., Nat. Immunol. 1: 311–316, 2000]. Because the immune response against cancer strongly depends on CTLs of Th1-like phenotype (Tc1), we verified here whether the kinetics of DCs activation also impacted on in vivo priming of tumor-specific CTLs. After pulsing with the CTL epitope TRP-2180–188, bone-marrow-derived DCs, exposed to lipopolysaccharide (LPS) for 8 h (8hDC), elicited a more powerful Tc1 response in C57BL/6 mice rather than did untreated DCs, or DCs exposed to LPS for 48 h (48hDC). Indeed, 8hDCs were the most potent protective and therapeutic vaccine against B16 melanoma. Despite a higher expression of MHC and costimulatory molecules by 48hDCs, 8hDCs and 48hDCs showed comparable allostimulatory and migration potential, and susceptibility to CTL-mediated apoptosis. However, 8hDCs exhibited a significantly higher interleukin (IL)-12 production potential. Release of IL-12 was necessary to induce potent Tc1 cells, because DCs from IL-12p40−/− mice, irrespective of their maturation level, generated low CTL responses, comparable with 48hDCs and 0hDCs from wild-type animals. Our data are relevant for the design of DC-based vaccines.

INTRODUCTION

It is generally accepted that, to fight intracellular pathogens and cancer, a robust CTL response is needed, which often requires help from CD4+ T cells (1). Vaccines designed to fight those diseases, therefore, are expected to induce potent antigen-specific CTLs. DCs are powerful APCs (2). In steady-state conditions, DCs reside within most body tissues, in which they uptake, process, and store large amounts of soluble and particulate antigens (2). Local inflammatory stimuli cause DCs to change the pattern of cytokines produced, increase cell surface expression of MHC and costimulatory molecules, and rapidly (within a few h) migrate to MIP-3β/SLC reach tissues (2, 3). Here, the interaction with CD4+ T cells causes a further step of DCs maturation (2), and allows them to provide CTLs with the “license to kill” (4). All of those behavioral changes transform DCs in natural adjuvants of the immune response (2).

DCs that are induced in vitro from CD34+ precursors (5, 6) can be loaded with the antigen of choice and can be re-infused into the donor’s tissues as potent anticancer vaccines (2).

Nevertheless, the issue of the state of activation of the DC to be used as vaccine is still under debate (2, 7). Indeed, DCs exposed to maturation stimuli appear to be more potent APCs than immature DCs (2, 7). It is likely however, that later during the development of an immune response, DCs are limited in their APC function to avoid the risks of excessive proliferation of selected lymphocyte clones, and/or development of autoimmune reactions. Kinetics studies have shown that, whereas recently activated DCs express cell surface molecules at high levels and produce relevant amounts of soluble factors, all required for T cell activation, DCs exposed for long periods to maturation stimuli undergo functional “paralysis” and/or “exhaustion” (8–10). Those functional and phenotypic changes directly affect the type of immune response induced. Langenkamp et al. (10), in particular, showed that, after in vitro activation by LPS, as well as by poly(I):poly(C), and TNF-α plus IL-1β, DCs produce IL-12, a cytokine directly involved in the generation of CTL responses (11), only transiently, with a peak between 5 and 8 h, and complete extinction after 18 h, the time at which they become refractory to further stimulation by CD40L. Similar kinetics has been found, using microarray techniques (12, 13). Even more interestingly, recently activated DCs (8hDC) preferentially induced Th1 responses, whereas exhausted DCs (48hDC) primed Th2 and nonpolarized T cells (10).

We investigated whether the kinetics of DC activation also impacted on in vivo priming of antigen-specific CTL responses. Bone-marrow-derived 8hDCs pulsed with TRP-2180–188 (14), and injected into C57BL/6 mice, elicited a stronger and more efficient melanoma-specific Tc1 response than did untreated DCs (0hDC) or 48hDCs. LPS-treated DCs exhibited similar in vitro survival and allostimulatory activity, as well as in vivo migration potential, but 8hDCs showed a significantly higher IL-12 production potential. Our in vivo findings strongly support the use of recently activated DCs for the generation of potent Tc1 responses and are, therefore, relevant for the design of DC-based vaccines.

MATERIALS AND METHODS

Mice, Tumor Cell Lines, and Reagents. Female mice, 8–10 weeks old, of the C57BL/6 (H-2b) wild-type (Charles River, Calco, Italy), MHC class II−/− (a gift of Dr. P. DellaBona, Istituto Scientifico H San Raffaele, Milan, Italy), and OT-1 (H-2d; a gift of Dr. Heath, Parkville, Victoria, Australia) strains, and BALB/c (H-2d, Charles River) wild type and IL-12p40−/− (IL-12−/−; a gift from Dr. L. Adorini, Bioxell, Milan, Italy) strains were housed in a specific pathogen free animal facility, and treated in accordance with the European Union guidelines, and with the approval of the Institutional Ethical Committee. The chemically induced EL-4 thymoma (H-2b), and the spontaneous B16F1 (H-2b, B16) melanoma (H-2b; B16) were purchased from American Type Culture Collection (Manassas, VA). Cell lines were cultured in RPMI 1640 supplemented with penicillin-streptomycin and 10% FCS (Eurolone, Wetherby West Yorkshire, United Kingdom). Unless specified, all chemical reagents were from Sigma-Aldrich, and mAbs were from BD PharMingen (San Diego, CA).
DCs Preparation and in vitro Functional Assays. DCs were prepared as described previously (15). Briefly, single-cell suspensions of bone-marrow were seeded into six-well plates at $2 \times 10^5$/ml in ISCOVE supplemented with penicillin-streptomycin and 10% FCS, and were cultured in RPMI 1640 containing 10% heat-inactivated FCS, with normal mouse serum for 15 min at 4°C, and double staining with the contamination (positive cultures were discarded). DCs were also incubated with Mycoplasma (kindly provided by P. Lane, Department of Immunology, University of Birmingham, Birmingham, GB) at a ratio 1:4 (10). CD40L-expressing J558 cells (kindly provided by P. Lane, Department of Immunology, University of Birmingham, Birmingham, GB) were added to DCs at a ratio 1:4. The labeled DCs were added to the target cells. The percentage of specific lysis was calculated as follows:

$$\text{Percent specific lysis} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Maximum cpm} - \text{Spontaneous cpm}} \times 100$$

**In Vivo Cytotoxicity Assay.** Naïve splenocytes were resuspended in PBS at a concentration of $10^7$/ml and were labeled with two different concentrations (1.25 and 0.125 µM) of 5- and 6-CFSE (Molecular Probes) according to the manufacturer’s indications. Cytotoxicity was calculated as (percentage CFSE+/negative × 100)/percentage CFSE+/positive. In each experiment, at least one naïve mouse was used to verify the survival of CFSE-labeled splenocytes 24 h after adoptive transfer.

In those animals, the cytolytic activity was always below 10%. 3C14 release of triplicates was calculated as follows:

$$\text{Average experimental cpm} - \text{Average spontaneous cpm} \times 100$$

**In Vivo and in vitro Polarization of Transgenic OT-1 Cells.** Lymph node cells from naïve OT-1 mice were injected at 10:1 ratio with OVA257-264-pulsed DCs with or without exogenous IL-12 (3 ng/ml; R&D Systems). Seven days later, cytokine production by effector cells was assessed by FACScan after stimulation for 4 h at 37°C with PMA (10^{-7} M) and ionomycin (1 µg/ml). B16-specific specific, respectively, and were expressed/10^6. 3C14 release of target cells alone (spontaneous release) was always <25% of maximal 3C14 release (target cells in 0.25 m HCl). LU were determined as the number of effector cells required for 30% (LU-30) and 10% (LU-10) B16-specific lysis, respectively, and were expressed/10^6.

**RESULTS**

8hDCs Elicit a More Powerful Protective and Curative Mela-noma-specific CTL Response Than 48hDCs. DCs propagated in vitro from bone-marrow precursors were exposed to LPS for the last 8 h.
8 or 48 h of culture. The different DC populations were thereafter pulsed with TRP-2_{180-188}, and injected i.d. into naïve C57BL/6 mice. When mice were challenged s.c. with 5 x 10^5 B16 cells (i.e., 10-fold the minimal tumorigenic dose; Ref. 18) a week after the third and last boost of DC-based vaccine, TRP-2_{180-188}-pulsed 8hDCs allowed survival of all B16-challenged mice until the end of the observation period (Fig. 1A). Conversely, only 40% of the mice vaccinated with TRP-2_{180-188}-pulsed 48hDCs survived tumor challenge, a result comparable with the one obtained vaccinating mice with 0hDCs (15). A statistically significant difference was found when the survival curves of mice vaccinated with 8hDCs and 48hDCs were compared by the log-rank test (P < 0.024). Vaccination with unpulsed DCs of all of the populations did not elicit relevant protective effects on tumor-challenged mice, when compared with animals that received injections of PBS (Fig. 1A and not shown).

We also compared our different DC-based vaccines in curative experimental settings (Fig. 1B). When vaccination started 5 days after melanoma cells injection, 75% of the animals receiving TRP-2_{180-188}-pulsed 8hDCs were still alive when all of the control animals were dead, and showed a remarkably prolonged survival. The difference between the survival curves of mice vaccinated with 8hDCs and 48hDCs was striking (P < 0.002). Indeed, treatment with peptide-pulsed 48hDCs caused only a slight and not statistically significant increase in animal survival when compared with the relative controls.

Of note, a statistically significant delay in melanoma growth was found when the survival curves of animals vaccinated with peptide-pulsed 0hDCs were compared with mice vaccinated with unpulsed 0hDCs (P < 0.005; not shown).

To assess the location and function of melanoma-specific CTLs induced by DC vaccination, we adopted an in vitro cytotoxicity assay (19). TRP-2_{180-188}-pulsed and unpulsed spleen cells from naïve mice, which were differentially labeled with different concentrations of CFSE, were i.v. injected into vaccinated mice 18 h before lymphoid organ collection and FACScan analysis (i.e., day 7). The highest cytolytic activity, measured by FACScan, was found between the results of the 48hDCs and 0hDCs. Six days later, mice were given i.v. injections of a mixture of CFSE-high-labeled, TRP-2_{180-188}-pulsed, and CFSE-low-labeled, unpulsed syngeneic spleen cells, as targets. Eighteen hours later, cells from draining lymph nodes were examined by FACScan. Histograms in A-D are representative of one mouse/group. Lower left angle of each panel, the mean ± SD of the cytotoxic activity detected in single animals in at least three independent experiments. Spleenocytes from the same animals were restimulated in vitro with TRP-2_{180-188} and were tested in a standard cytotoxicity assay against B16 (●) and unpulsed (□) or TRP-2_{180-188}-pulsed (■) EL-4 cells. Data are means ± SD of triplicates of the percentages of specific 51Cr release at the indicated E:T ratios. E-H, the data from one representative experiment of at least three experiments, all of which showed the same pattern.

**Fig. 1.** Comparison of the protective and curative effects of 8hDCs and 48hDCs in the B16 melanoma model. A, C57BL/6 mice were immunized by three weekly i.d. injections of one of the following vaccines: PBS (●), 8hDCs or 48hDCs (■) and not shown, respectively; 5 animals/group unpulsed (w/o) or pulsed (10 animals/group) with TRP-2_{180-188} (TRP-2; ■ and ●, respectively). One week after the last boost, mice were challenged s.c. by 5 x 10^5 B16 cells. Values are expressed as percentage of surviving animals at a given time. Statistical comparison, conducted by the log-rank test, of the survival curves gave the following results: 8hDCs+TRP-2 versus 8hDCs w/o, P < 0.0006; 8hDCs+TRP-2 versus 48hDCs+TRP-2, P < 0.0241; 8hDCs+TRP-2 versus PBS, P < 0.0001; 48hDCs+TRP-2 versus 8hDCs w/o, P < 0.017; 48hDCs+TRP-2 versus 48hDCs w/o, P < 0.012; and 48hDCs+TRP-2 versus PBS, P < 0.005. In all other conditions, the comparison was not statistically significant. B, in curative experiments, mice were given s.c. injections of 5 x 10^5 B16 cells. Five days later, mice were randomly assigned to one of the following treatments: three weekly i.d. injections of PBS (●; n = 10), 8hDCs or 48hDCs unpulsed (w/o; □ and not shown, respectively; 5 animals/group) or pulsed with TRP-2_{180-188} (TRP-2; ■ and ●, respectively; 10 animals/group). Animals were evaluated as above. Statistical analyses: 8hDCs+TRP-2 versus 8hDCs w/o, P = 0.0009; 8hDCs+TRP-2 versus 48hDCs+TRP-2, P < 0.0019; and 8hDCs+TRP-2 versus PBS, P < 0.0003. In all other conditions, the comparison was not statistically significant. Both A and B report the cumulated data of two independent experiments.

**Fig. 2.** 8hDCs elicit a stronger melanoma-specific CTL response than do 48hDCs. Mice were given one-time i.d. injections of 2 x 10^6 TRP-2_{180-188}-pulsed 8hDCs (A and E), 48hDCs (B and F), or 0hDCs (C and G), or PBS (D and H). Six days later, mice were given i.v. injections of a mixture of CFSE-high-labeled, TRP-2_{180-188}-pulsed, and CFSE-low-labeled, unpulsed syngeneic spleen cells, as targets. Eighteen hours later, cells from draining lymph nodes were examined by FACScan. Histograms in A-D are representative of one mouse/group. Lower left angle of each panel, the mean ± SD of the cytotoxic activity detected in single animals in at least three independent experiments. Spleenocytes from the same animals were restimulated *in vitro* with TRP-2_{180-188} and were tested in a standard cytotoxicity assay against B16 (●) and unpulsed (□) or TRP-2_{180-188}-pulsed (■) EL-4 cells. Data are means ± SD of triplicates of the percentages of specific 51Cr release at the indicated E:T ratios. E-H, the data from one representative experiment of at least three experiments, all of which showed the same pattern.

*RECENTLY ACTIVATED DCs AND IL-12*
B16, a melanoma endogenously expressing TRP-2 (17), was found to be 3–7-fold higher in splenocytes from 8hDCs-injected mice (LU-10: 152 × 10^6; Fig. 2E), when compared with splenocytes from mice vaccinated with 48hDCs (LU-10: 55 × 10^6; Fig. 2F) and 0hDCs (LU-10: 23 × 10^6; and Fig. 2G), respectively. As reported previously (15), splenocytes from mice that received PBS injections (Fig. 2H), unpulsed DCs, or peptide in PBS (not shown) did not kill any target used.

The direct involvement of CD8+ T cells in the immune response induced by the different vaccinations was demonstrated by the findings that depletion of CD8+ cells by mAbs, one day before CTL assays, completely abolished the antigen-specific CTL activity in all of the lymphoid organs examined (cytolytic activity, 7 ± 3.2%; n = 3).

The immune response induced in vivo against TRP-2 by different vaccines is predominantly CD4 dependent (e.g., 18). To better define the immune response elicited by TRP-2-pulsed 8hDCs, vaccinations were performed in MHC class II−/− mice as well as in mice depleted of CD4+ cells 1 day before the injection of CFSE-labeled cells. No cytolytic activity was detected in the lymphoid organs of vaccinated MHC class II−/− mice (i.e., 5 ± 2.4%; n = 3). Because depletion of CD4+ cells during the effector phase of the immune response generated by 8hDCs did not alter the antigen-specific CTL activity (i.e., 82 ± 6.2%; n = 3), our data confirm that the immune response induced by such vaccine is also CD8 mediated and CD4 dependent.

8hDCs Skew the Antigen-specific CTL Response toward a Tc1 Response. The above reported data strongly underline the relevance of the kinetics of DC activation in the induction of potent tumor-specific CTL responses and lend weight to the hypothesis that in vivo recently activated DCs also skew the immune response toward a Tc1 response. To evaluate the cytokine production profile of CTLs activated in vivo by 8hDCs, we took advantage of the highly cytotoxic transgenic OT-1 cells, specific for OVA257-264 (20). Purified CD8+ cells from OT-1 mice were labeled with CFSE and were injected i.v. into recipient mice 1 day before i.d. injection of peptide-pulsed DCs. When draining lymph node cells were analyzed by FACSscan 3 days after vaccine injection, the highest percentage of proliferating OT-1 cells was found in mice vaccinated with 8hDCs (>85%; Fig. 3A–C). Mice vaccinated with 48hDCs (Fig. 3D–F) or 0hDCs (Fig. 3G–I) showed a significantly lower percentage of proliferating OT-1 cells (<60 and 50%, respectively). Animals given injections with PBS showed a marginal proliferation (25%; Fig. 3L–N). Even more strikingly, in mice vaccinated with 8hDCs, almost 50% of the proliferating cells produced IFN-γ (Fig. 3A), the paradigmatic type 1 cytokine (21). Conversely, <10% of the CD8+ cells produced IFN-γ in all of the other groups of vaccinated mice (Fig. 3, D, G, and L). We also measured IL-4 and IL-10 production by in vivo activated OT-1 cells. Irrespective of the experimental condition examined, we did not find significant and consistent production of IL-4 (Fig. 3B, E, H, and M) and IL-10 (Fig. 3C, F, I, and N). These data, therefore, strongly suggest that on vaccination with recently activated DCs, a potent Tc1 immune response is generated.

The Ability of 8hDCs to Generate Tc1 Cells Does Not Depend on Their Phenotype, Allostimulatory and Migration Potential, and Susceptibility to CTL-mediated Apoptosis. We sought to identify which factors were responsible for the high immunogenic potential of 8hDCs. When the different DC populations were analyzed by FACSscan for surface expression of molecules involved in antigen presentation and T-cell activation, we found that 8hDCs expressed higher levels of MHC class I, class II, CD80, CD86, and CD40 molecules when compared with 0hDCs (Fig. 4). Of note, the latter showed a heterogeneous expression of the indicated markers, suggesting that 0hDCs were a mixed population of immature and matured DCs. However, 8hDCs expressed similar if not lower levels of all of the indicated markers when compared with 48hDCs, therefore excluding the possibility that the higher immunogenic potential of 8hDCs versus 48hDCs depends on the amount of MHC and costimulatory molecules expressed by the two DC populations.

Despite different levels of expression of MHC and costimulatory molecules, 8hDCs and 48hDCs exerted similar allostimulatory activi-
ity in vitro. Indeed, in standard 2 and 3 days, MLR overlapping proliferative responses were measured at all DC:effector ratios (Fig. 5 and not shown). As expected, 0hDCs stimulated an alloreactive response only at the highest DC:effector ratios.

Another possible explanation for the higher efficiency of 8hDCs in inducing Tc1 immune responses when compared with 48hDCs could have been a different migration potential. To address this issue, 10^6 cells for each DC population were labeled with the dye CMFDA and were injected into naïve C57BL/6 mice. Twenty-four h later, cells from popliteal lymph nodes were labeled also with anti-CD11c-PE and analyzed by FACSscan. In 2 independent experiments, which gave similar results, a higher percentage of CMFDA^+ CD11c^+ cells was found in popliteal lymph nodes from mice that received injections of 8hDCs (0.17 ± 0.05; n = 3) and 48hDCs (0.23 ± 0.1; n = 3) when compared with mice that received injections of 0hDCs (0.04 ± 0.02, n = 3). A difference statistically significant was found between 0hDCs and 8hDCs (P < 0.01) or 48hDCs (P < 0.03), but not between 8hDCs and 48hDCs. Hence, the higher immunogenic potential of 8hDCs could not be attributed to a better migration potential.

Once migrated to the lymphoid organs, DCs may be the target of antigen-specific CTLs (22). Therefore, we analyzed whether our DC populations were differently susceptible to CTL-mediated activation of caspases, one of the most proximal events in perforin-dependent apoptosis. Twenty-four h after incubation with naïve OT-1 cells in the presence or absence of OVA_{257–264}, cells were stained with CD11c-Cy, CD86-FITC mAbs, and Caspataq red caspase, and were analyzed by FACSscan. In all of the DC populations tested, the presence of OVA_{257–264} did not induce a substantial increase in caspase activation over the background (i.e., absence of the peptide): 0hDCs, 17.5 ± 2.9 and 20.7 ± 2.2%; 8hDCs, 15.2 ± 2.9 and 22.5 ± 2.8%; and 48hDCs, 13.8 ± 1.9 and 18.9 ± 1.8, respectively, making highly unlikely the hypothesis of an in vivo CTL-mediated selection in favor of the 8hDCs.

The Ability of 8hDCs to Generate Tc1 Cells Depends on Their IL-12 Production. Because IL-12 is a key cytokine for the induction of type 1 T cells (11), we verified whether a difference in IL-12 production could be found between 8hDCs and 48hDCs. IL-12p70 release was assessed by ELISA in the culture supernatants at the end of the period of exposure to LPS (i.e., day 7). Irrespective of the time at which LPS was added to the culture, DCs produced equal amounts of IL-12p70 (8hDC, 172 ± 33 pg/ml; 48hDCs, 187 ± 27 pg/ml; n = 3) that were much higher than those produced by 0hDCs (46 ± 3.5 pg/ml, n = 3; 8hDCs versus 0hDCs, P < 0.015; 48hDCs versus 0hDCs, P < 0.006).

Elisa tests, however, do not define the time at which IL-12 has been produced. Neither do they allow quantification of IL-12-producing DCs, nor take into account the fact that DCs can consume IL-12 produced during the culture. We, therefore, also evaluated the DC pattern of cytokine expression during LPS exposure by intracellular staining followed by FACSscan analysis. In eight independent experiments we found that, different from 0hDCs, which only marginally produced IL-12 (1.5 ± 0.8%), 23.7 ± 5.1% of the 8hDCs were positive for IL-12 (Fig. 6, A, and D; DCs not exposed to LPS versus 8hDCs, P < 0.0001). As expected, a prolonged exposure to LPS dramatically reduced the percentage of IL-12-producing DCs (12.1 ± 1.9; 8hDCs versus 48hDCs, P < 0.0004; Fig. 6, A, D, and G). Conversely, we did not find any different production of IL-2 and IL-10, nor production of IFN-γ and IL-4 by the DC populations analyzed (not shown). Comparable results were obtained using CD40L as promaturation stimulus (i.e., IL-12^-^ cells: 0hDCs, 4 ± 2%; 8hDCs, 16.1 ± 1.5%; 48hDCs, 3.2 ± 1%; n = 3). Conversely, TNF-α alone did not induce a level of IL-12 production significantly higher than the one found in 0hDCs (not shown).

To verify to what extent IL-12 was involved in the induction of
potent Tc1 cells, we generated DCs from wild-type and IL-12<sup>−/−</sup> BALB/c mice. The susceptibility to LPS of DCs from BALB/c and C57BL/6 mice was comparable. Indeed, the expression of MHC and costimulatory molecules by 8hDCs from wild-type and IL-12<sup>−/−</sup> BALB/c mice was similar, and intermediate between 0hDCs and 48hDCs (not shown). 8hDCs from wild-type animals were the most efficient producers of IL-12 (Fig. 6, B, E, and H). As expected, none of the DC populations from IL-12<sup>−/−</sup> mice were not able to produce IL-12 (Fig. 6, C, F, and I). When BALB/c mice were vaccinated with the different populations of gp70<sub>233–431</sub>-pulsed DCs from wild-type and IL-12<sup>−/−</sup> mice, the highest level of cytotoxicity against peptide-pulsed splenocytes was found in the lymphoid organs of mice vaccinated with 8hDCs from wild-type animals (Fig. 6L). Vaccination with 8hDCs from IL-12<sup>−/−</sup> mice strongly reduced the induction of antigen-specific CTL (wild-type versus IL-12<sup>−/−</sup> 8hDCs, P < 0.007), therefore, suggesting that IL-12 was a key factor for the induction of antigen-specific Tc1 cells by 8hDCs. That finding was further sustained by in vitro experiments of T-cell polarization (Fig. 6M). Indeed, when naïve OT-1 cells were cultured with OVA<sub>257–264</sub>-pulsed DCs in the absence of exogenous IL-12, 8hDCs allowed induction of a much higher percentage of IFN-γ-producing cells than did 48hDCs and 0hDCs (43 ± 2.8, 4.9 ± 1.2, and 2.1 ± 0.4, respectively). Conversely, all DC populations were comparably able to polarize toward Tc1 cells when exogenous IL-12 was added to the culture medium.

**DISCUSSION**

Our results demonstrate that recently activated DCs are more powerful cancer vaccines than similar DC populations that are not exposed to LPS or that are exposed to LPS for a prolonged period of time. This conclusion is sustained by the finding that vaccination with 8hDCs generated a far more potent protective and curative antigen-specific immune response than vaccination with 0hDCs or 48hDCs. This immune response was systemic, CD8 mediated, and CD4 dependent. Moreover, only 8hDCs caused the induction of antigen-specific Tc1 cells. Finally, the demonstration that 8hDCs from IL-12<sup>−/−</sup> mice lost the capacity to induce strong CTLT cell immune responses and behaved like the other DC populations, strongly supports the key role of IL-12 in the in vivo induction of potent Tc1 cells (23).

The kinetics of IL-12 production by 8hDCs allows DCs to reach lymphoid tissues at a time in which that cytokine is maximally produced (3). Here, 8hDCs should rapidly induce CD4<sup>+</sup> T cell activation (3), possibly Th1 polarization (24), and even faster priming of potent CTLs (25, 26). That narrow window of time should avoid the risk of overactivation of the immune response and/or the activation of bystander, and possibly autoreactive, T cells. Indeed, 48hDCs, although equally efficient in migrating to the draining lymph nodes, have lost most of their potential to induce powerful type 1 responses.

Th1 cells can be induced by different DC populations and/or in the presence of different promaturation stimuli (27). Indeed, in the presence of strong viral stimuli, or low DC:T-cell ratios, even DC2 cells can promote Th1 cell induction (27). The conditions favoring the induction of Tc1 cells are less characterized. We favor the hypothesis that, irrespective of the DC population involved, strong promaturation stimuli (e.g., LPS, bacterial CpG DNA, and double-stranded viral DNA) allow rapid and intense production of IL-12 as well as other Th1-prone factors. At later time points during the DC life span, exhaustion, probably in the presence of reduced amounts of antigen and low DC:T-cell ratios, may favor induction of Tc2 and nonpolarized T cells.

Langenkamp et al. (10) showed that “exhausted” DCs were more prone to prime Th2 and nonpolarized T cells. In in vitro experiments, we also found that 48hDCs preferentially induced nonpolarized CD8<sup>+</sup> T cells. Furthermore, exhausted in the presence of IL-4 were the best inducers of Th2 cells. In vivo, 48hDCs were not able to induce a detectable Tc1 immune response. Experiments are ongoing to verify whether Th2 cells are induced under those conditions.

It has been previously described (e.g., in Refs. 15 and 28) that DCs that are cultured in vitro in the presence of GM-CSF and IL-4 (i.e., 0hDCs) and pulsed with synthetic peptides are able to generate variable protective and therapeutic effects, depending on the cancer model used. Others (29) have shown that those 0hDCs exerted minimal if any therapeutic activity. Bone-marrow-derived DCs obtained by in vitro cultures in the presence of GM-CSF (and IL-4) usually exhibit different levels of maturation (e.g., in Refs. 5 and 30), which often depend on their manipulation. Indeed, it is well known that simple pipetting or transferring of DC cultures may cause their rapid maturation (5, 30, 31). Hence, one explanation for the above reported discrepancies may be the different grade of spontaneous maturation of the DC preparations. Other possible explanations may be the different source of DCs, culture conditions, and vaccination schedule.

The effect of the kinetics of IL-12 production on DC activation was measured at time points earlier than 8 h (i.e., 15 min and 1, 2, and 4 h). We found that the percentage of IL-12<sup>+</sup> DCs sharply increased 15 min after adding LPS to the culture, remained at similar levels for the following 4 h, and reached a peak at 8 h. A rapid decline in IL-12<sup>+</sup> DCs was found between 24 and 48 h. The kinetic of expression of cell-surface molecules (e.g., IA-b, CD80, and CD86) was slower, with a relevant increase at 8 h or later. Therefore, we suggest that, also for mouse DCs, the more favorable window of time to obtain activated, highly IL-12-producing DCs is between 6 and 8 h after LPS stimulation.

After preclinical studies in mice, the first vaccinations in humans were conducted with DCs not exposed to promaturation stimuli (reviewed in Ref. 2). More recently, cancer patients have been immunized with DCs exposed to promaturation stimuli for at least 24 h (32, 33). It would be interesting to verify whether recently activated DCs are better cancer vaccines in humans also.

In conclusion, we show that recently activated DCs skew in vivo the antigen-specific immune response toward a Th1 phenotype. This information is of relevance in designing DC-based vaccines. Indeed, a potent Tc1 immune response is highly desirable in the first phases of a tumor-specific immune response, when powerful CTLs are needed to eliminate all malignant cells.

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