Immortalized Dendritic Cell Line Fully Competent in Antigen Presentation Initiates Primary T Cell Responses In Vivo

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Summary

Dendritic cells (DC) can provide all the known costimulatory signals required for activation of unprimed T cells and are the most efficient and perhaps the critical antigen presenting cells in the induction of primary T cell–mediated immune responses. It is now shown that mouse cell lines with many of the features of DC can be generated using the MIB-2-N11 retroviral vector transducing a novel envAKR-myc^H' fusion gene. The immortalized dendritic cell line (CB1) displays most of the morphologic, immunophenotypic, and functional attributes of DC, including constitutive expression of major histocompatibility complex (MHC) class II molecules, costimulatory molecules B7/BB1, heat stable antigen, intracellular adhesion molecule 1, and efficient antigen-presenting ability. Granulocyte/macrophage colony–stimulating factor (GM-CSF) proved to be effective in increasing MHC class II molecule expression and in enhancing presentation of native protein antigens. In comparison with macrophages, CB1 dendritic cells did not exhibit phagocytic and chemotactic activity in response to various stimuli and lipopolysaccharide activation was ineffective in inducing tumor necrosis factor α or interleukin 10 production. CB1 cells, pulsed with hapten in vitro and injected into naïve mice were able to induce delayed-type hypersensitivity responses, further increased with pretreatment with GM-CSF, indicating that these cells may represent an immature, rather than a mature DC. The ability of CB1 to prime T cells in vivo could provide a tool to design novel immunization strategies.

Abbreviations used in this paper: CI, chemotaxis index; CS, contact sensitivity; DC, dendritic cell; DNBS, 2,4-dinitro benzene sulfonic acid; DTH, delayed-type hypersensitivity; fmlp, formyl peptide; HSA, heat stable antigen; ICAM-1, intracellular adhesion molecule 1; MCP-1, macrophage chemotactic protein 1; MIP-1α, macrophage inflammatory protein 1α; PAF, platelet-activating factor; PI, phagocytosis index; rm, recombinant mouse; SpWMb, sperm whale myoglobin.

Dendritic cells (DC), first described by Steinman and Cohn in 1973 (1), are a population of widely distributed leukocytes that play a key role in the immune system (2, 3) given that they are: (a) highly specialized in antigen presentation; (b) the principal activators of resting T cells in vitro (4, 5); (c) the major source of immunogenic epitopes for specific T cell clones after administration of antigen in vivo (6, 7) and (d) the most potent initiators of primary T cell–mediated responses in vivo (8).

Several studies (2, 3) have suggested that DC provide naïve T cells with all the necessary signals required for activation and proliferation. These signals are generated by the interaction of complexes of MHC molecules and antigenic peptides with the TCR (9), and by the engagement of costimulatory molecules, including binding of B7/BB1 molecules on APC to CD28 receptor on the T cell surface (10, 11). The first signal alone elicits effector functions only in activated T cells and is unable to stimulate naïve or resting T cells, which in the absence of costimulatory signals can enter a period of unresponsiveness (12–14). The expression of the costimulatory molecule B7/BB1 on DC populations has been recently reported and shown to be critical in DC-driven primary T cell responses (15–17).

Understanding the mechanisms underlying the potent stimulatory capacities of DC could explain how T cells are primed and how the immune response is initiated. With this knowledge one might try to manipulate immune responses at very early stages and provide a way for inducing immunity or tolerance. However, an important limitation in the study of DC biology has been the small numbers of cells
available from any tissue, given that no stable cell lines that are clearly similar to DC have been obtained so far. Three different tissues have been used as major sources of DC: mouse spleen, the epidermis, where DC are known as Langerhans cells, and human blood. In each case DC constitute a tiny fraction of the starting tissue, representing about 1% of crude spleen (18) or epidermal (19, 20) cell suspensions and 0.1–1% of PBMC (21). More recently, Inaba et al. (22) have described a method for generating DC from both peripheral blood and bone marrow precursors, but cell proliferation ceases within 1–3 wk.

A different approach is to generate cell lines of DC. Earlier studies from our laboratory (23, 24) have shown that, using a mixture of recombinant retroviruses transducing the avian v-myc oncoprotein, the immortalization of macrophages could be readily achieved from several different tissues. The immortalized and cloned macrophages were shown to respond in a physiological way to activation signals and were able to exert all the expected functions of macrophages (25).

The retroviral recombinant genome was molecularly cloned and transfected into the ψ2 packaging cell line. The novel MIB-2-N11 helper-free retrovirus was then used to infect mouse spleen primary cultures (Sassano, M., F. Granucci, P. Paglia, M. Foti, and P. Ricciardi-Castagnoli, manuscript submitted for publication). With this approach, immortalized DC lines were also generated. One clone, named CB1, was extensively characterized and shown to possess many of the features described for DC, including the expression of B7/B81, heat stable antigen (HSA), intracellular adhesion molecules 1 (ICAM-1), and the reactivity with several anti-DC mAbs.

The stimulatory capacity of the CB1 clone was shown in vitro, both in primary MLR and in antigen-specific T cell proliferative assay. GM-CSF increased the constitutive MHC class II molecule expression and was essential for antigen-specific APC activity in vitro. In addition, CB1 cells pulsed in vitro with haptoenzymes were able to induce a delayed-type hypersensitivity (DTH) immune response, thus indicating that T cell priming can be achieved using an antigen-loaded cell line as natural adjuvant.

Materials and Methods

Generation of Dendritic Cell Lines. Spleen cell suspensions were prepared from newborn DBA/2 mice (Charles River, Como, Italy) in RPMI-1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FCS (GIBCO BRL, Gaithersburg, MD), glutamine, penicillin-streptomycin, and 0.5 mM β-ME. RBC were lysed and spleen cells were seeded at 10^6/ml density in 35-mm petri dishes. Immortalization was carried out with the MIB-ψ2-N11 retroviral vector generated by transfection of the packaging cell line ψ2 with a molecularly cloned recombinant retrovirus (Sassano, M. et al., manuscript submitted for publication). This recombinant retrovirus was originally obtained by transfection of mouse fibroblasts with the avian MH2 and the mouse AKRv viral genomes and resulted in recombinant retroviruses carrying the gag, pol, env genes and LTR of AKRv and the avian v-myc oncopogene (23–25).

Infection with MIB-ψ2-N11 was performed with filtered supernatant from a 24-h subconfluent culture of the viral producer cell line ψN11 diluted 1:1 with complete medium containing 10 μg/ml polybrene (Sigma Chemical Co.). After 1 h incubation at 37°C in a 5% CO2 incubator, half volume of fresh medium was added and then regularly changed twice a week. During the first week after infection, cells were fed with 10% L929.6C-conditioned media, reduced at 5% in the following 2 wk, and than gradually eliminated.

About 20–30 d after infection, multiple foci of dividing cells were observed in the petri dishes. Cultures could then be trypsinized and cells replated. The cell line was considered established after 10 passages, and then cloned by limiting dilution.

Immunostaining Analysis of CBI Cells. Cell surface expression of leukocyte markers was assessed using the following mAbs: M1/9.3 (TIB 122), RA.3.3.A1 (TIB 146), F4/80 (HB198), 2.4G2 (HB 197), FD 4418 (TIB 213), M1/70 (TIB 128), M1/42 (TIB 126), 33D1 (TIB 227); NLDC145, B5.5, RB6–8C5, B21–2, M1/69 (kindly provided by R. M. Steinman, The Rockefeller University, New York), and FA11, 3D6 (kindly provided by S. Gordon, Oxford University, Oxford, UK), IG10 (PharMingen, San Diego, CA), followed by anti-rat Ig-FITC (Boehringer Mannheim, Mannheim, Germany); N418 (HB224), 145–2Cl1, 3E2, H57–597 (PharMingen), followed by anti–hamster Ig-PE (Southern Biotechnology Associates, Birmingham, AL), biotinylated MK-D6, 39–10–8, AMS-16, IM7, RM2–5, RM4–5, 53–6–7, RA.3–682, 34–2–12 (PharMingen) mAbs, followed by Streptavidin–PE (Sigma Chemical Co.). Purified Igs (PharMingen) or unrelated isotype-matched Abs were used as controls.

CBI cells (5 x 10^5) were incubated for 30 min at 4°C with hybridoma supernatants, affinity purified or biotinylated primary mAbs followed by either anti-rat Ig–FITC or anti–hamster Ig–PE, or Streptavidin–PE. For MHC class II expression, cells were stained before and after 48-h treatment with either 100 U/ml of recombinant mouse (rm) IFN-γ or 200 ng/ml of rmGM-CSF (Genzyme Corp., Cambridge, MA).

Expression was performed on FACSscan® cytofluorimeter (Becton Dickinson & Co., Mountain View, CA) after dead cells removal by propidium iodide gating.

 Peroxidase immunostaining of CBI cells was carried out to show reactivity for intracellular antigens recognized by the rat anti-DC mAbs 2A1 (26) and by the hamster anti-DC M342 (27) kindly provided by N. Romani (University of Innsbruck, Innsbruck, Austria). CBI cells were grown on coverslips and fixed in cold acetone. Cells were incubated for 1 h with mAbs in the presence of 1% normal mouse serum, washed and stained with peroxidase mouse anti–hamster ( Pierce, Rockford, IL) or anti–rat Ig (Boehringer Mannheim), and visualized using diaminobenzidine as substrate (Sigma Chemical Co.).

Adherence, Phagocytosis, Chemotaxis, and Cytokine Production. Phagocytic ability was assessed using zymosan particles either untreated or opsonized with normal mouse serum. Phagocytosis index (PI) was evaluated at the microscope. Chemotaxis index (CI) was kindly measured by P. Sacerdote (University of Milan) in response to 10^-7 M formyl peptide (fmlp), 10 ng/ml macrophage chemotactic protein (MCP-1) or 10^-10 M platelet activating factor (PAF). TNF-α secretion was measured by ELISA according to the suggestions of the supplier (Genzyme Corp.). IL-1β secretion was kindly measured by P. Gnecchi (Farmitalia-Carlo Erba, Nerviano, Italy) with a RIA method previously described (28).

Northern Blot Analysis. Northern blot analysis of mRNAs from CBI cells and a positive control was carried out using a specific mψ-myc probe previously described (25). The mψ-Myc 3' probe was derived from a chicken genomic library and it represents the 3' region of the avian myc gene which does not cross-hybridize with the murine myc genes.
Expression of B7/BB1 Molecule. mRNA (polyA)' from LPS-activated splenocytes, LPS-activated A20 cells, and untreated CB1 cells was prepared from 5 x 10^6 cells. Activation was carried out for 72 h with 10 μg/ml of LPS (Escherichia coli, B5:055; Sigma Chemical Co.). First strand cDNA synthesis was obtained using AMV reverse transcriptase (Promega Corp., Madison, WI) and an oligo dT primer. PCR was performed in a 50-μl vol using 2.5 μl cDNA, 5 μl 10× PCR buffer (Perkin Elmer Corp., Norwalk, CT), 1.5 mM MgSO_4, 0.25 mM dNTPs, 1.5 U Taq polymerase (Boehringer Mannheim), and 25 pmol forward and reverse oligonucleotide primer. Nucleotides 249-266 and 1153-1169 of the B7/BB1 cDNA sequence were used as sense and antisense oligonucleotides, respectively, as previously described (15). PCR conditions were 40 cycles using a thermocycler (Perkin-Elmer Corp.): 1 min, 95°C; 45 s, 56°C; 1 min, 72°C. PCR products were analyzed by agarose gel electrophoresis and blotted to nylon membrane. Southern blot was probed with an internal B7/BB1 oligonucleotide (nucleotides 661-680) as described (15).

In Vitro T Cell Stimulatory Capacity of CB1 Cells. In an antigen-specific presentation assay, 10^5 CB1 or MT2 cells, treated with either 100 U/ml IFN-γ or 200 ng/ml rmGM-CSF, or A20 cells, were used as APC for sperm whale myoglobin (SpWMb, 0-5 AM) to 10^5 antigen-specific T hybrid cells (13.26.8 H6.1 SpWMb-specific H-2d restricted T cells were kindly provided by A. M. Livingstone, Basel Institute of Immunology, Basel, Switzerland). Antigen, at the indicated doses, was present during all the time of the assay. After 24 h of incubation, 100 μl of supernatant was transferred and tested for the IL-2 content on the HT.2 cell line (5 x 10^5 cells/well). Proliferation was measured using the MTT assay. In a MLR assay, GM-CSF-treated or untreated dendritic CB1 cell line and MT2/1 macrophage cell line (23) were treated with mytomycin C (4 μg/ml) and used, at the indicated doses, as stimulators in coculture with 3 x 10^5 allogeneic C57BL/6 T cells as responders. Synthetic T cells were also used as controls. T cells were the nonadherent spleen cells purified on nylon wool columns. [3H]Tdr incorporation (2.5 μCi/ml) was measured 72 h later.

Induction of Contact Sensitivity. Contact sensitivity was induced by injection of 2,4-dinitro benzene sulfonic acid (DNBS; Eastman Kodak, Rochester, NY)-modified or FITC-modified CB1 cells pretreated or not with GM-CSF (200 ng/ml). CB1 cells were derivatized with either 200 μg/ml of FITC (Sigma Chemical Co.) or with 1 mg/ml DNBS for 30 min at 37°C as described (29, 30) and inoculated subcutaneously into the dorsal skin of DBA/2 mice. Each mouse received 10^5-10^6 cells suspended in HBSS in a final vol of 250 μl. Recipient mice and nonsensitized controls were ear challenged 5 d later with either 25 μg FITC or 15 μg of 2,4-dinitro-1-fluorobenzene (DNFB, Sigma Chemical Co.) on the side of both ears. Mice sensitized epicutaneously were painted with 150 μg DNFB or 2 mg FITC on the shaved abdomen, as described (29, 30). Ear thickness was measured with an engineer's micrometer.

Results

Generation of Dendritic Cell Lines. Spleen cell suspensions from newborn mice were infected with the retroviral vector MIB-2-N11. About 3 wk after infection, foci were observed and proliferating cells, detaching from adherent aggregates, were easily cloned. Among several clones obtained, the clone named CB1 was selected because of its dendritic morphology. Fig. 1 shows typical aggregates of proliferating CB1 cells with, at the periphery of the clusters, cells possessing a dendritic appearance. Characteristically, these cells display sheetlike processes, with a striking motility when observed at video microscopy (data not shown). This behavior is not exhibited by other leukocytes and may have a functional significance in the survey of T cells (2, 3).

The estimated doubling time of CB1 cell line was about 20 h (data not shown). Productive viral infection of CB1 cells with the MIB-2-N11 retroviral vector (described in Fig. 2 a) was tested by Northern blot analysis using a specific mycMHZ2 probe (31): genomic and subgenomic transcripts of the expected size were detected (Fig. 2 b).

In addition to spleen, other tissues have been infected with the MIB-2-N11 retroviral vector. From infected cultures of bone marrow and skin tissues DC-like cell lines were generated (Paglia, P., manuscript in preparation). Moreover, from

Figure 1. Morphological appearance of the cell line CB1 (phase contrast microscopy). Clusters of CB1 cells 48 h after seeding: at the periphery of the clusters, cells with a dendritic phenotype are observed.

Figure 2. (a) The MIB-2-N11 proviral genome. Viral immortalization with the MIB-2-N11 retroviral vector: Northern blot analysis of CB1 cells (A) and of a positive control (B) probed with a specific mycMHZ2 probe. (b) The genomic and subgenomic transcripts with the expected size (arrowheads).
BALB/c spleen cultures, one additional dendritic cell line (D2SC/1) was established and extensively characterized (Paglia, P., manuscript in preparation), thus showing the reproducibility of the method.

**CBI Cell Line Has Many of the Features of Splenic DC.** Cytofluorometric cell surface analysis with several mAbs (Fig. 3) and intracellular staining with the M342 (27) and the 2A1 (26) (Fig. 4) revealed a characteristic DC immunophenotype of the CB1 cell clone. CB1 cells express high levels of MHC-I molecules, of the common leukocyte antigen CD45, the adhesion molecules Pgp-1 (CD44) and CD11c, the latter shown to be a typical marker of spleen DC (32). A bright staining was also achieved with mAbs against B7/BB1, HSA, and ICAM-1 costimulatory molecules. Intermediate levels of expression were detected for 33D1, NLDC-145, FA11 antigens and LFA-1 and MAC-1 integrins. Moreover, CB1 cells lack the B cell marker B220 and are negative for T cell–specific determinants: Thy1, CD3-e, TCR, CD4, and CD8. These results are in agreement with those reported for spleen DC (26) and Langerhans cells (20, 33). Low levels of F4/80 and Fcy-RII were present on CB1 cell surfaces; however, comparing CB1 cells with macrophage MT2 cells we found that expression of F4/80 and Fcy-RII was fivefold higher on MT2 cells (data not shown). Finally, we detected high CD2 expression on CB1 cell surface. This result was unexpected and is intriguing because it suggests that homotypic interactions between DC and T cells may occur.

MHC-II expression on CB1 cells treated or untreated with GM-CSF was tested using a panel of anti–MHC mAbs recognizing various epitopes of I-A or I-E molecules. These mAbs (MK-D6, B21-2, 39-10-8, and AMS-16) detected constitutive expression of MHC-II molecules on CB1 cells with a high degree of variability (from low to high) depending on the mAb used (data not shown). Interestingly, in all cases the expression of MHC-II molecules was enhanced when CB1 cells were treated with GM-CSF. In contrast, IFN-γ did not exert any influence on MHC-II expression on CB1 cells, whereas it was effective in inducing both I-A and I-E expression on MT2 macrophage cell line (data not shown).

Taken as a whole these results suggest that CB1 cells may represent an immature splenic DC precursor still responsive to GM-CSF.

**Functional Properties of CB1 Cells.** Spleen DC are loosely and transiently adherent to plastic surfaces (1-3); similarly,
CB1 cells were loosely adherent and pipetting was enough to detach them. Compared to macrophages (MT2/1 cells) which can be detached only in the presence of trypsin, CB1 cells were very much less adherent (Table 1).

Phagocytic and chemotactic activities were also tested. DC are known to be not actively phagocytic (2, 3) and, consistent with this, CB1 cells exhibited very little zymosan phagocytosis, whereas MT2/1 macrophages were able to migrate in response to all stimuli (Table 1). This finding is of particular interest since it suggests that DC have chemotactic and migratory properties different from those of macrophages.

Controversial results have been reported concerning the production of cytokines by different DC. Spleen DC have been shown to produce little or no IL-1 when activated with a variety of stimuli (34). In contrast, epidermal Langerhans cells have been identified as the major source of mRNA for IL-1β and macrophage inflammatory proteins, MIP-1α and MIP-2, among unstimulated and hapten-activated epidermal cells, with a strong upregulation of the former upon short-term culture (35–38). In contrast to MT2/1 macrophages, CB1 cells were shown to produce very small amounts of TNF-α and IL-1β upon stimulation with LPS (Table 1), confirming at a clonal level previous negative results on IL-1 expression by enriched spleen DC preparations (34).

Expression of the Costimulatory Molecule B7/BB1. APC need to provide two different signals in order to activate T lymphocytes: an antigen-specific interaction and accessory nonspecific signals. Recently, the costimulatory B7/BB1 molecule has been identified on APC (10, 11, 39, 40) and has proven to be essential for proliferation of unprimed allogeneic T cells in MLR assay and CD4+ T cells in antigen-specific proliferative responses (15, 16).

Spleen DC express constitutively the B7/BB1 molecule (15, 16) whereas the A20 cell line or B cells require activation by LPS or through cross-linking of either surface MHC class II molecules (41) or their cytoplasmic domain (11).

Transcription of the B7/BB1 gene was evaluated in LPS-activated splenocytes, in A20 cells, and in CB1 DC. PCR analysis was carried out using sense and antisense oligonucleotides derived from the murine B7/BB1 cDNA sequence (40, 42). PCR products are shown in the upper part of Fig. 5. Only CB1 cells have a constitutive transcription of the B7/BB1 gene whereas in A20 cells and in splenocytes transcription was induced only after treatment with LPS. To show specificity, PCR products were also analyzed in Southern blots (Fig. 5, bottom) probed with an internal B7/BB1 oligonucleotide. Cell surface expression of the protein was confirmed by cytofluorimetric analysis using the 1G10 mAb (11) which recognizes the mouse B7/BB1 molecule (Fig. 3).

Table 1. Adherence, Phagocytosis, Chemotaxis, and Cytokine Production by CB1 and MT2/1 Cell Lines

| Functions                     | Stimuli | Cell line | Activity |
|-------------------------------|---------|-----------|----------|
| Adherence to plastic surface  | MT2/1   | + + +     |
|                               | CB1     | +         |
| Phagocytosis                  | Zymosan | MT2/1     | + + + (89 PI) |
|                               |         | CB1       | + / - (5 PI) |
| Chemotaxis                    | fmlp    | MT2/1     | + (3.6 CI) |
|                               |         | PAF       | + + (6.7 CI) |
|                               |         | MCP-1     | + + + (11 CI) |
|                               | fmlp    | CB1       | -         |
|                               | PAF     | -         |
|                               | MCP-1   | -         |
| Cytokine production           | LPS     | MT2/1     | TNF-α (3.0 ng/ml) |
|                               |         |           | IL-1β (5.0 ng/ml) |
|                               | LPS     | CB1       | TNF-α (0.4 ng/ml) |
|                               |         |           | IL-1β (0.2 ng/ml) |

Figure 5. Expression of B7/BB1. (Top) Analysis of PCR products from untreated (A) or LPS-activated splenocytes (B), untreated (C), or LPS-activated A20 cells (D), and untreated CB1 cells (E). (Bottom) Southern blot analysis of PCR products hybridized with an internal B7/BB1 oligonucleotide. LPS-activated splenocytes (B), LPS-activated A20 cells (D) and untreated CB1 cells (E).
A strong stimulating activity of DC in the primary MLR has been known since 1978 (45). We compared the stimulatory capacity of CB1 and MT2/1 macrophages in a primary dose-response MLR assay. As shown in Fig. 7, CB1 cells possess accessory function in the in vitro allogeneic T cell proliferative assay. This response does not have the expected amplitude typical of fully differentiated DC, but this may again reflect the immature state of CB1 cells. Antigen-pulsed DC, but not other APC such as B cells or macrophages, can be administered in vivo to prime T lymphocytes without additional adjuvants. DC are considered the most potent initiators of various primary T cell-mediated responses in vivo such as contact sensitivity (CS) (46), allograft rejection (8, 47), and activation of MHC-restricted T cells (6, 7). Moreover, it has been shown that antigen-pulsed DC are able to induce an Ab response in vivo (48). The ability of CB1 cells to prime

Discussion

The potential use of elements of the immune system to prime naive animals offers great advantages over standard immunizations with artificial adjuvants. The "dirty trick of immunologists", as stated by Charles A. Janeway, Jr. (50), which consists of the addition of mineral oil or killed bacteria (CFA) to highly purified or recombinant protein antigens to ini-

Figure 7. In vitro T cell stimulatory capacity of CB1 cells: MLR assay. Graded doses of CB1 (●●) and MT2/1 (○○) cells were used as stimulators in coculture with 3 × 10^3 allogeneic C3HBL/6 T cells as responders. Incubation of 3 × 10^3 CB1 cells with 3 × 10^3 syngeneic T cells resulted in 550 cpm [H]ThdR uptake (background level).

Figure 8. Induction of contact sensitivity by injection of DNBS- or FITC-modified CB1 cells. 10^4-10^6 CB1 cells were derivatized with either FITC or with DNBS for 30 min at 37°C, and inoculated subcutaneously into the dorsal skin of DBA/2 mice. Recipient mice and nonsensitized controls were ear challenged 5 d later with either FITC or DNFB on each side of both ears. Mice sensitized epicutaneously were painted with DNFB or with FITC on the shaved abdomen. Ear thickness was measured with an engineer's micrometer immediately before challenge and 24, 48, and 72 h after challenge. Data are expressed as the change (from prechallenge levels) in ear thickness × 10^-4 inches and represent the mean maximal increase within the 72-h observation period (n = 4).
Induction of contact sensitivity by injection of DNBS- or FITC-modified CB1 cells (50,000) activated with GM-CSF. The procedure is the same as in Fig. 8, except that CB1 cells were pretreated or not for 48 h with 200 ng/ml GM-CSF before hapten derivatization. Now we know that unprimed T lymphocytes recognize antigenic epitopes expressed on the cell surface of DC which possess all the costimulatory signals (e.g., B7/BB1, ICAM-1, LFA-3, and HSA) necessary for T cell proliferation and differentiation (2, 3, 5, 11). Moreover, priming with antigen-pulsed DC can also elicit a memory immune response. DC can thus be considered, as suggested by Ralph Steinman, "nature's adjuvants" (2, 12) that can initiate in vivo both cell-mediated (DTH) and T cell–dependent Ab responses (3, 48).

In this paper we show the generation of a DC line using a novel retroviral vector, MiBv2-N11, transducing an activated env^KR-mycMH2 fusion gene. The established DC line, CB1, was shown to possess many of the phenotypic features of lymphoid DC, including expression of the B7/BB1, HSA, and ICAM-1 molecule.

The GM-CSF inducibility of class II expression in CB1 cells might mean that these cells represent a DC precursor, rather than a mature DC. The observation that bone marrow and blood DC precursors derive from Ia^- cells (26, 44) supports this hypothesis. In addition, immature DC, including freshly isolated epidermal Langerhans cells, express lower levels of class II molecules compared to a fully differentiated DC (2, 3, 51).

Functionally, CB1 exhibited both the ability of processing and presenting native protein antigens in vitro as well as the function of sensitizing T cells in vivo, as assessed by the ability to initiate a primary DTH response.

GM-CSF–increased expression of MHC class II molecules was necessary for the in vitro antigen-specific APC activity of CB1 cells, and enhanced the ability of CB1 cells to induce CS, confirming a key role of GM-CSF in the differentiation and activation of DC accessory functions. GM-CSF was also shown to support the growth and differentiation of DC from mouse blood or bone marrow cultures (22) and of early human cord blood and bone marrow progenitors into DC (51). In addition, GM-CSF in cooperation with TNF-α, promotes the in vitro differentiation of epidermal Langerhans cells into mature DC (2, 3, 52), and induces generation of Langerhans cells from human cord blood CD34+ precursors (53).

Moreover, our results add further evidence to the notion that DC and macrophages can be distinguished for a variety of properties, including morphology, immunophenotype, phagocytosis, response to chemotactic stimuli, cytokine production profile, and cytokine activation requirements. It has been shown that APCs produce a limited number of predominant immunogenic peptides and that DC make the most immunogenic ligands for antigen-specific TCR (2, 3). In this regard, the CB1 cell line could be used to probe the immunogenicity of various antigens among which tumor-associated antigens are the most relevant candidates.

The stimulatory function of the DC line CB1 could also provide a tool to design novel immunization strategies. The role played by the route of administration, the organ where antigen presentation occurs, and the type of immune response elicited are questions that can now be addressed using in all experiments the same clonal population of DC.

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References

1. Steinman, R.M., and Z.A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. J. Exp. Med. 137:1142.

2. Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol. 9:271.

3. Romani, N., and G. Schuler. 1992. The immunologic properties of epidermal Langerhans cells as a part of the dendritic cell system. Springer Semin. Immunopathol. 13:265.

4. Inaba, M.D., M.D. Witmer, and R.M. Steinman. 1984. Clustering of dendritic cells, helper T lymphocytes, and histocompatible B cells during primary antibody responses in vitro. J. Exp. Med. 160:858.

5. Croft, M., D.D. Duncan, and S.L. Swain. 1992. Response of naive antigen-specific CD4+ T cells in vitro: characteristics and antigen-presenting cell requirements. J. Exp. Med. 176:1431.

6. Inaba, K., J.P. Metlay, M.T. Crowley, and R.M. Steinman. 1990. Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific MHC-restricted T cells in situ. J. Exp. Med. 172:631.

7. Crowley, M., K. Inaba, and R.M. Steinman. 1990. Dendritic cells are the principal cells in mouse spleen bearing immunogenic fragments of foreign proteins. J. Exp. Med. 172:383.

8. Lechler, R.I., and J.R. Batchelor. 1982. Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. J. Exp. Med. 155:31.

9. Davis, M., and P.J. Bjorkman. 1988. T cell antigen receptor genes and T cell recognition. Nature ( Lond.). 334:395.

10. Young, J.W., L. Koulова, S.A. Soergel, E.A. Clark, R.M. Steinman, and B. Dupont. 1990. The B7/BB1 antigen provides one of several costimulatory signals for the activation of CD4+ T lymphocyte by human blood dendritic cells in vitro. J. Clin. Invest. 90:229.

11. Nabavi, N., J.C. Freeman, A. Gault, D. Godfrey, M.L. Nadler, and L.H. Glimcher. 1992. Signalling through the MHC class II cytoplasmic domain is required for antigen presentation and induces B7 expression. Nature (Lond.). 360:266.

12. Inaba, K., and R.M. Steinman. 1985. Protein-specific helper T lymphocyte formation initiated by dendritic cells. Science (Wash. DC). 229:475.

13. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. An accessory cell–derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. J. Immunol. 142:2617.

14. Tan, P., C. Anasetti, J.A. Hansen, J. Melrose, M. Brunvand, J. Bradshaw, J.A. Ledbetter, and P.S. Linsley. 1993. Induction of alloantigen-specific hypersensitivity in human T lymphocytes by blocking interaction of CD28 with its natural ligant B7/BB1. J. Exp. Med. 177:165.

15. Larsen, C.P., S.C. Ritchie, T.C. Pearson, P.S. Linsley, and R.P. Lowry. 1992. Functional expression of the costimulatory molecule, B7/BB1, on murine dendritic cell populations. J. Exp. Med. 176:1215.

16. Symington, F.W., W. Brady, and P.S. Linsley. 1993. Expression and function of B7 on human epidermal Langerhans cells. J. Immunol. 150:1286.

17. Liu, Y., B. Jones, W. Brady, C.A. Janeway, and P.S. Linsley. 1992. Co-stimulation of murine CD4 T cell growth: cooperation between B7 and heat-stable antigen. Eur. J. Immunol. 22:2855.

18. Steinman, R.M., G. Kaplan, M.Z. Witmer, and A. Cohn. 1979. Identification of a novel cell type in peripheral lymphoid organs of mice. J. Exp. Med. 149:1.

19. Schuler, G., and R.M. Steinman. 1985. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. J. Exp. Med. 161:526.

20. Romani, N., A. Lenz, H. Glassel, H. Stossel, U. Stanzl, O. Majdic, P. Frisch, and G. Schuler. 1989. Cultured human Langerhans cells resemble lymphoid dendritic cells in phenotype and function. J. Invest. Dermatol. 93:600.

21. Freudenthal, P.S., and R.M. Steinman. 1990. The distinct surface of human blood dendritic cells, as observed after an improved isolation method. Proc. Natl. Acad. Sci. USA. 87:7698.

22. Inaba, K., R.M. Steinman, M.W. Pack, H. Aya, M. Inaba, T. Sudo, S. Wolpe, and G. Schuler. 1992. Identification of proliferating dendritic cell precursors in mouse blood. J. Exp. Med. 175:1157.

23. Ricciardi-Castagnoli, P., and P. Paglia. 1992. New tools investigating macrophage differentiation. Res. Immunol. 143:101.

24. Righi, M., M. Sassano, P. Valsassini, S. Shammah, and P. Ricciardi-Castagnoli. 1991. Activation of the M-CSF gene in mouse macrophages immortalized by retroviruses carrying v-myc oncogene. Oncogene. 6:803.

25. Pirani, L., B. Stockinger, S. Betz Corradin, M. Sironi, M. Sassano, P. Valsassini, M. Righi, and P. Ricciardi-Castagnoli. 1991. Mouse macrophage clones immortalized by retroviruses are functionally heterogenous. Proc. Natl. Acad. Sci. USA. 88:7546.

26. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Maramatsu, and R.M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. 176:1693.

27. Agger, R., M. Witmer-Pack, N. Romani, H. Stossel, W.J. Swiggard, J.P. Metlay, E. Storozynsky, P. Freimuth, and R.M. Steinman. 1992. Two populations of splenic dendritic cells detected with M342, a new monoclonal to an intracellular antigen of interdigitating dendritic cells and some B lymphocytes. J. Leukocyte Biol. 52:34.

28. Gnocchi, P., C. Losa, D. Trizio, and A.M. Isetta. 1992. Development and applications of a radioimmunoassay (RIA) for in vitro and in vivo quantification of murine IL-1β. Lymphokine Cytokine Res. 2:257.

29. Macatonia, S.E., A.J. Edwards, and S.C. Knight. 1986. Dendritic cells and the initiation of contact sensitivity to fluorescein isothiocyanate. Immunol. 59:509.

30. Girolomoni, G., S. Pastore, V. Zacchi, A. Cavanis, A. Marconi, and A. Giannetti. 1993. Phosphatidyserine enhances the ability of epidermal Langerhans cells to induce contact hypersensitivity. J. Immunol. 150:4236.

31. Righi, M., A. Pierani, A. Boglia, G. De Libero, L. Morì, V. Marini, and P. Ricciardi-Castagnoli. 1989. Generation of new oncogenic murine retroviruses by cotransfection of clone AKR and MH2 proviruses. Oncogene. 4:223.

32. Metlay, J., M.D. Witmer-Pack, R. Agger, M.T. Crowley, D. Lawless, and R.M. Steinman. 1990. The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. J. Exp. Med. 171:1753.

33. Romani, N., S. Koide, M. Crowley, M. Witmer-Pack, A.M. Livingstone, C.G. Fathman, K. Inaba, and R.M. Steinman. 1989. Presentation of exogenous protein antigens by dendritic cells to T cell clones. J. Exp. Med. 169:1169.

34. Koide, S., and R.M. Steinman. 1987. Induction of murine interleukin 1: stimuli and responsive primary cells. Proc. Natl. Acad. Sci. USA. 84:3802.
35. Heufler, C., G. Topar, F. Koch, B. Trockenbacher, K. Kämpgen, N. Romani, and G. Schuler. 1992. Cytokine gene expression in murine epidermal cell suspensions: interleukin 1β and macrophage inflammatory protein 1α are selectively expressed in Langerhans cells but are differentially regulated in culture. J. Exp. Med. 176:1221.

36. Matsue, H., P.D. Cruz, Jr., P.R. Bergstresser, and A. Takashima. 1992. Langerhans cells are the major source of mRNA for IL-1β and MIP-1α among unstimulated mouse epidermal cells. J. Invest. Dermatol. 99:537.

37. Schreiber, S., O. Kilgus, E. Payer, R. Kuttil, A. Elbe, C. Mueller, and G. Stingl. 1992. Cytokine pattern of Langerhans cells isolated from murine epidermal cell cultures. J. Immunol. 149:3524.

38. Enk, A., and S.I. Katz. 1992. Early molecular events in the induction phase of contact sensitivity. Proc. Natl. Acad. Sci. USA. 89:1398.

39. Gimmi, C.D., G.J. Freeman, J.G. Gribben, K. Sugita, A.S. Freeman, C. Morimoto, and L.M. Nadler. 1991. B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. Proc. Natl. Acad. Sci. USA. 88:6575.

40. Linsley, P.S., P.M. Wallace, J. Johnson, M.G. Gibson, J.L. Greene, J.A. Ledbetter, C. Singh, and M.A. Tepper. 1992. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. Science (Wash. DC). 257:792.

41. Koulova, L., E.A. Clark, G. Shu, and B. Dupont. 1991. The CD28 ligand B7/BBI provides costimulatory signal for activation of CD+ T cells. J. Exp. Med. 173:759.

42. Freeman, G.J., G.S. Gray, C.D. Gimmi, D.B. Lombard, L.J. Zhou, M. White, J.D. Fingeroth, J.C. Gribben, and L.M. Nadler. 1991. Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. J. Exp. Med. 174:625.

43. de Bruijn, M.L.H., J.D. Nieland, C.V. Harding, and C.J. Melief. 1992. Processing and presentation of intact hen egg-white lysozyme by dendritic cells. Eur. J. Immunol. 22:2347.

44. Inaba, K., M. Inaba, M. Deguchi, K. Hagi, R. Yasumizu, S. Ikehara, S. Muramatsu, and R. Steinman. 1993. Granulocytes, macrophages and dendritic cells arise from a common major histocompatibility complex class II-negative progenitor in mouse bone marrow. Proc. Natl. Acad. Sci. USA. 90:3038.

45. Steinman, R.M., and M. Witmer. 1978. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. Proc. Natl. Acad. Sci. USA. 75:5132.

46. Britz, J.S., P.W. Askensase, W. Ptak, R.M. Steinman, and R.K. Gershon. 1982. Specialized antigen-presenting cells: splenic dendritic cells and peritoneal-exudate cells induced by mycobacteria activate effector T cells that are resistant to suppression. J. Exp. Med. 155:1344.

47. Larsen, C.P., R.M. Steinman, M. Witmer-Pack, D.F. Hankins, P.J. Morris, and J.M. Austrin. 1990. Migration and maturation of Langerhans cells in skin transplant and explants. J. Exp. Med. 172:1483.

48. Sornasse, T., V. Flamand, G. De Becker, H. Bazin, F. Tielemans, K. Thielemans, J. Urbain, O. Leo, and M. Moser. 1992. Antigen-pulsed dendritic cells can efficiently induce an antibody response in vivo. J. Exp. Med. 175:15.

49. Girolomoni, G., J.C. Simon, P.R. Bergstresser, and P.D. Cruz, Jr. 1990. Freshly isolated spleen dendritic cells and epidermal Langerhans cells undergo similar phenotypic and functional changes during short-term culture. J. Immunol. 145:2820.

50. Janeway, C.A., U. Dianzani, P. Portoles, S. Rath, E.P. Reich, J. Rojo, Y. Yagi, and D.B. Murphy. 1989. Cross-linking and conformational change in T cell receptors: role in activation and in repertoire selection. Cold Spring Harbor Symp. Quant. Biol. 54:657.

51. Reid, C., A. Stackpoole, A. Meager, and J. Tikeripe. 1992. Interactions of tumor necrosis factor with granulocyte-macrophage colony-stimulating factor and other cytokines in the regulation of dendritic cell growth in vitro from early bipotent CD34+ progenitors in human bone marrow. J. Exp. Med. 174:2681.

52. Koch, F., C. Heufler, E. Kämpgen, D. Schneeweiss, G. Böck, and G. Schuler. 1990. Tumor necrosis factor α maintains the viability of murine epidermal Langerhans cells in culture, but in contrast to granulocyte/macrophage colony-stimulating factor, without inducing their functional maturation. J. Exp. Med. 171:159.

53. Caux, C., C. Dezutter-Dambuyant, D. Schmitt, and J. Ban- chereau. 1992. GM-CSF and TNFα cooperate in the generation of dendritic Langerhans cells. Nature (Lond.) 360:258.