STABLE LINES OF GENETICALLY MODIFIED DENDRITIC CELLS FROM MOUSE EMBRYONIC STEM CELLS

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Background. **The capacity to activate naïve T cells sets dendritic cells (DCs) apart from other antigen-presenting cells, making them attractive targets for immune intervention during deleterious immune responses. The inherent resistance of terminally differentiated DCs to conventional strategies for genetic modification has, however, greatly limited our understanding of the molecular mechanisms underlying their function.**

Methods and Results. **We report the derivation of long-term cultures of untransformed DCs, uniformly expressing a defined mutant phenotype by the directed differentiation of cloned embryonic stem cells, stably transfected with a reporter gene. Introduction of the gene encoding enhanced green fluorescent protein into pluripotent stem cells demonstrated no observable impact on the phenotype, immunogenicity, or capacity for maturation of DCs differentiated from them.**

Conclusions. **The production of unlimited numbers of mutant DCs from genetically modified embryonic stem cells paves the way for the systematic elucidation of gene function in this cell type and the rational design of DCs for use in immunotherapy.**

Although it is widely accepted that dendritic cells (DCs) play a pivotal role in the induction of all immune responses (*1*), little is known of the molecular basis underlying their unique properties. Such uncertainty has hindered the application of DCs to the clinic for the treatment of cancer (*2*), autoimmunity (*3*), and allograft rejection (*4*) and has been compounded by difficulties inherent in the genetic modification of DC ex vivo. Approaches to transfection based on the use of lipids or cationic peptides as vehicles for the delivery of heterologous DNA have achieved stable expression in a small proportion of cells (*5*) but require strategies for their subsequent purification. Viral vectors have, in contrast, achieved higher transduction efficiencies but may adversely affect DC physiology, as evidenced by their impact on maturation. Whereas some viral vectors arrest DCs at an immature stage, others provoke their premature maturation (*6*), preventing the analysis of gene function during successive phases of their life cycle. To address these issues, we developed a novel approach to DC biology that exploits the unique properties of embryonic stem (ES) cells: their pluripotency, self-renewal, and tractability for genetic modification.

The spontaneous differentiation of ES cells maintained in suspension gives rise to embryoid bodies (EBs) composed of cell types derived from each of the embryonic germ layers. This process mimics early ontogeny including development of the visceral yolk sac, thereby providing a microenvironment permissive for hematopoiesis (*7*). We recently reported how the addition of granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-3 to cultures of EBs directs the differentiation of hematopoietic progenitors toward the DC lineage, producing an almost limitless source of myeloid DCs (*8*). These cells fail to mature spontaneously in culture, remaining immature for prolonged periods of time. Nevertheless, in response to bacterial products such as lipopolysaccharide (LPS), ES cell-derived DCs (esDCs) acquire a mature phenotype and potently activate naïve T cells. We show how a reporter gene introduced into the parent ES cell line using a standard, nonviral approach is expressed by more than 90% of the resulting DCs without perturbing their maturation, immunogenicity, or migration patterns in vivo. Our results demonstrate how this novel approach to DC biology may facilitate the design of DC with desirable properties for use in immunotherapy.

MATERIALS AND METHODS

The derivation and maintenance of the CBA/Ca ES cell line ESF116 has been described (*8*). Before transfection, ES cells were seeded into gelatinized six-well plates at 10^5 cells per well in medium supplemented with 1,000 U/mL of recombinant leukemia inhibitory factor and cultured for 48 hr to promote adherence. ESF116 was transfected with a plasmid conferring neomycin resistance and containing the gene encoding enhanced green fluorescent protein (EGFP) under control of the elongation factor-1α promoter. Transfection was performed using LipofectAMINE Plus (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. After 48 hr, ES cells were selected in 600 μg/mL of the neomycin analog G418, and the resulting transfectants were cloned in gelatinized 96-well flat-bottomed plates. Individual clones were expanded and screened for expression of EGFP by flow cytometry.

To promote differentiation, clones of transfected ESF116 were cultured on bacteriologic plastic (4×10^5 cells/dish) for 14 days in the absence of recombinant leukemia inhibitory factor but in the presence of a "holding dose" of 300 μg/mL of G418. The resulting EBs were plated onto tissue culture plastic in medium supplemented with 200 IU/mL of IL-3 (R&D Systems, Minneapolis, MN), 25 ng/mL of GM-CSF, and 300 μg/mL of G418. After 5 days, lightly adherent esDCs could be observed around the perimeter of the EBs and became confluent over the ensuing 2 weeks. Immature esDCs were harvested by gentle pipetting, leaving behind the adherent EBs. Replating overnight encouraged the adherence of any contaminating stromal cells, enriching the DC fraction, as described (*9*). Addition of 1 μg/mL of LPS induced the release of esDCs from the tissue culture plastic; mature esDCs were, therefore, enriched by harvesting nonadherent cells (*9*).

Mature esDCs were stained with the following biotinylated monoclonal antibodies: HB32 (H-2E^k), 1G10 (CD80), and GL-1 (CD86; PharMingen, San Diego, CA). Antibody binding was visualized using streptavidin-APC, and samples were analyzed with a Becton Dick-

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inson FACSCalibur (Franklin Lakes, NJ). Multiplex polymerase chain reaction was performed in triplicate on a Prism 7700 (Perkin Elmer, Foster City, CA) using primers and probes specific for CCR7 (5'-GCTGCGTCAACCCTTTCTTG-3', 5'-ACCGACGCGTTCCGTACAT-3', and 5'FAM-TTCATCGGCGTCAAGTTCCGC-3') and hypoxanthine phosphoribosyltransferase (5'-GACCGGTCCCGTCATGC-3', 5'-TCATAACCTGGTTCATCATCGC-3', and 5'VIC-ACCCGCAGTCCCAGCGTCGTG-3'). Mixed leukocyte reactions were performed using nylon wool-purified T cells from syngeneic or C57Bl/10 mice, as previously described (*8*). To trace the migration of esDCs in vivo, 7-μm sections were cut from the draining lymph nodes of mice receiving 3×10^6 cells in the footpad. EGFP$^+$ cells were localized within tissues by counterstaining for CD4 (clone RM4–5; PharMingen) followed by Texas Red conjugated donkey anti-rat immunoglobulin (Jackson Laboratory, Bar Harbor, ME).

RESULTS AND DISCUSSION

Genetic modification of terminally differentiated DCs has proven difficult to achieve without corrupting the very properties that make them unique. To address this issue, we investigated whether genes introduced into an ES cell line would be faithfully expressed by their DC progeny. We used LipofectAMINE Plus (Life Technologies) to transfect ESF116 with a construct conferring neomycin resistance and containing the gene encoding EGFP under the elongation factor-1α promoter. Transfected ESF116 were selected in G418, and multiple clones were established expressing EGFP at high levels, four of which were chosen for further study. A representative clone, E3, readily formed EBs during 14 days of culture under selection conditions. When plated onto tissue culture plastic in medium supplemented with GM-CSF and IL-3, 90% of EBs adhered and formed colonies composed of the variety of differentiated cell types documented previously (*7*). In addition, each sustained the differentiation of up to 10^7 immature DCs, which rapidly regenerated every 4 to 5 days after routine harvesting. Figure 1A shows characteristic clusters of esDCs around the perimeter of a typical EB (asterisk) after 7 days of culture, which can be seen to express high levels of EGFP by inverted fluorescence microscopy. Flow cytometric analysis of esDCs harvested from such cultures revealed that more than 96% were inherently fluorescent compared with their counterparts differentiated from nontransfected ES cells (Fig. 1B). In eight independent experiments, the level of transgene expression averaged 93.4%±4.8%. Similar results were obtained on differentiation of each of the three alternative ES cell clones. Culture of EGFP$^+$ esDCs for 5 days in the absence of G418 caused no detectable loss of fluorescence intensity compared with cells maintained in selection (Fig. 1C), demonstrating the stability of the mutant phenotype.

To determine whether introduction of the reporter gene into ESF116 had compromised subsequent DC function, esDCs were cultured overnight in medium containing 1 μg/mL of LPS. Under such conditions, esDCs rapidly matured as evidenced by the translocation of major histocompatibility complex class II to the cell surface (Fig. 2A) and up-regulation of CD80 and CD86 (Fig. 2B,C), a phenotype indistinguishable from that of esDCs from nontransfected ES cells (*8*). Furthermore, CCR7 was dramatically up-regulated on maturation, as assessed by real-time polymerase chain reaction (Fig. 2D). Under confocal microscopy, these cells displayed the dendritic morphology typical of mature DCs (Fig. 2E). Most important, however, coculture of this population

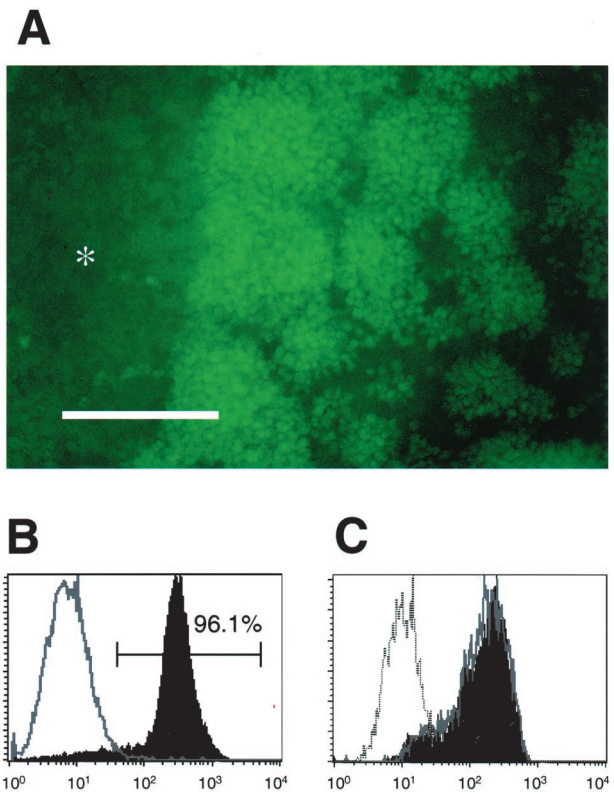

FIGURE 1. Analysis of enhanced green fluorescent protein (EGFP) expression by dendritic cells (DCs) derived from genetically modified embryonic stem (ES) cells. (A) Low-power fluorescence micrograph of clusters of EGFP$^+$ ES cell-derived DCs (esDCs) surrounding a single embryoid body (EB), only a portion of which is shown (*asterisk*). Bar is 100 μm. (B) Flow cytometric analysis of EGFP$^+$ esDCs (*filled histogram*) compared with control esDCs differentiated from nontransfected ES cells (*open histogram*). (C) Fluorescence intensity of EGFP$^+$ esDCs cultured for 5 days in the absence of G418 (*filled histogram*) compared with that of cells maintained in parallel under selection conditions (*open histogram*). The level of background fluorescence emitted by esDCs differentiated from nontransfected control ES cells (*dotted line*).

with purified splenic T cells from allogeneic C57Bl/10 mice provoked potent primary responses, substantially greater than those of control syngeneic T cells (Fig. 2F), confirming their immunocompetence in vitro.

To investigate the behavior of genetically modified esDCs in vivo, we administered 3×10^6 cells in the footpad of syngeneic mice and sectioned the draining lymph nodes 24 hr later. Whereas in multiple experiments, immature esDCs could not be detected in the lymph nodes of injected mice, those receiving mature esDCs consistently contained cells endogenously expressing EGFP. Double-labeling experiments revealed their capacity to interdigitate among CD4$^+$ T cells (Fig. 2G), consistent with the high expression of CCR7, known to confer on mature DCs their propensity to migrate to the T-cell areas of secondary lymphoid tissues (*10*). In time-course experiments, esDCs expressing the transgene persisted in vivo for at least 6 days.

The routine generation of stable lines of untransformed DCs expressing a defined mutant phenotype would have a

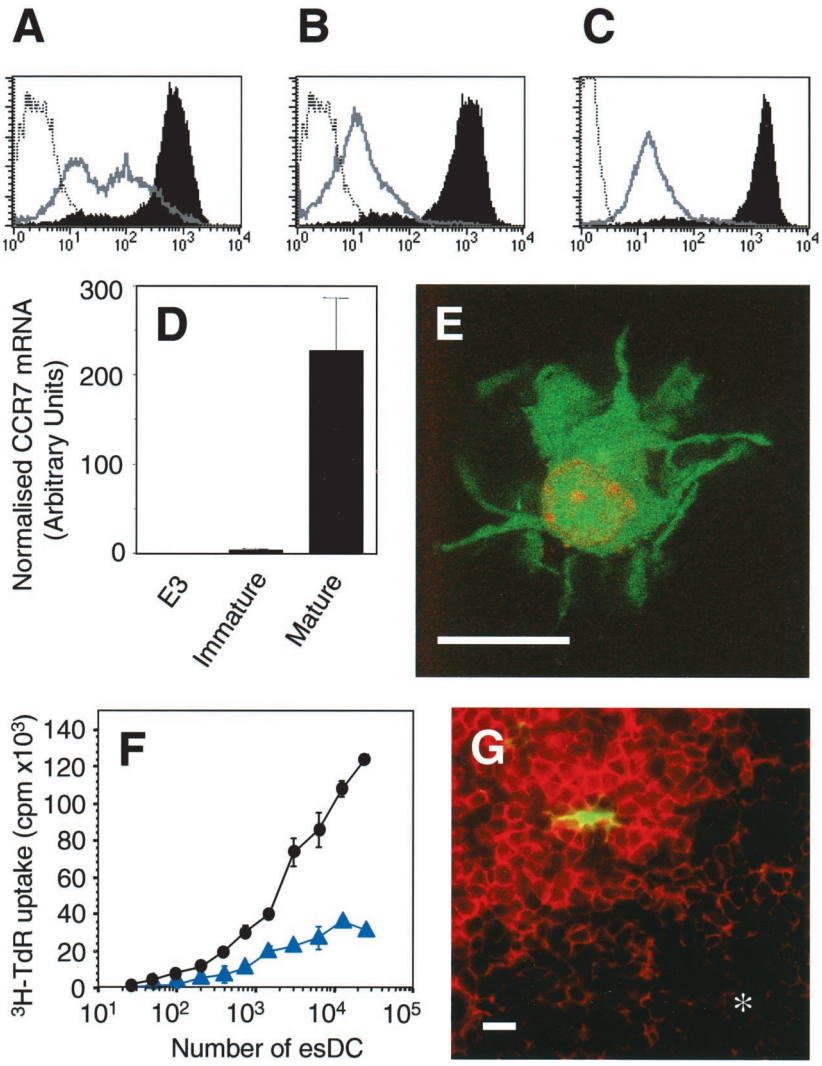

FIGURE 2. Phenotypic and functional maturation of EGFP$^+$ esDCs in response to lipopolysaccharide (LPS). (A-C) Phenotypic analysis of EGFP$^+$ esDCs before (*open histograms*) or after (*filled histograms*) overnight exposure to LPS showing up-regulation of major histocompatibility complex class II (A), CD80 (B), and CD86 (C). The level of background staining using species- and isotype-matched control monoclonal antibodies (*dotted lines*). (D) Real-time polymerase chain reaction showing expression of CCR7-specific mRNA by the ESF116 transfectant E3 and both mature and immature esDCs differentiated from it. cDNA from LPS-treated bone marrow-derived DCs was used to generate a standard curve to calibrate the threshold cycle. (E) High-power confocal micrograph of EGFP$^+$ esDCs counterstained with TOPRO-3, a dye specific for nucleic acids that preferentially stains nucleoli (*red*). Bar is 10 μm. (F) Immunogenicity of EGFP$^+$ esDCs on coculture with naïve, allogeneic T cells (*black circles*). The level of syngeneic responses among naïve CBA/Ca T cells (*blue triangles*). (G) Representative section from the draining lymph node of a mouse receiving mature esDCs in the footpad. A typical EGFP$^+$ cell (*green*) is shown interdigitating between CD4$^+$ T cells (*red*). The adjacent B cell area (*asterisk*). Bar is 10 μm.

significant impact on the fields of functional genomics and immunotherapy. Whereas the most successful nonviral approaches to transfection have achieved gene expression in no more than 35% of DCs (*5*), our results show how selection and cloning of genetically modified ES cells permits the generation of pure populations of immunocompetent DCs uniformly expressing the transgene. DCs derived in this way are stably immature under normal culture conditions but remain responsive to maturation stimuli, permitting the systematic elucidation of gene function at successive stages of their life cycle. Our current studies are aimed at exploiting this novel technology within the field of transplantation by overexpressing immunoinhibitory receptors and dominant negative forms of costimulatory molecules anticipated to undermine the capacity of DCs to prime for allograft rejection, favoring, instead, a tolerogenic phenotype (*3*, *4*). Most important, the advent of human ES cells (*11*) holds considerable promise for extending such studies to the rational design of human DCs with therapeutic potential.

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