Directed differentiation of dendritic cells from mouse embryonic stem cells

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Dendritic cells (DCs) are uniquely capable of presenting antigen to naive T cells, either eliciting immunity [1] or ensuring self-tolerance [2]. This property identifies DCs as potential candidates for enhancing responses to foreign [3] and tumour antigens [4], and as targets for immune intervention in the treatment of autoimmunity and allograft rejection [1]. Realisation of their therapeutic potential would be greatly facilitated by a fuller understanding of the function of DC-specific genes, a goal that has frequently proven elusive because of the paucity of stable lines of DCs that retain their unique properties, and the inherent resistance of primary DCs to genetic modification. Protocols for the genetic manipulation of embryonic stem (ES) cells are, by contrast, well established [5], as is their capacity to differentiate into a wide variety of cell types *in vitro*, including many of hematopoietic origin [6]. Here, we report the establishment, from mouse ES cells, of long-term cultures of immature DCs that share many characteristics with macrophages, but acquire, upon maturation, the allostimulatory capacity and surface phenotype of classical DCs, including expression of CD11c, major histocompatibility complex (MHC) class II and co-stimulatory molecules. This novel source should prove valuable for the generation of primary, untransformed DCs in which candidate genes have been overexpressed or functionally ablated, while providing insights into the earliest stages of DC ontogeny.

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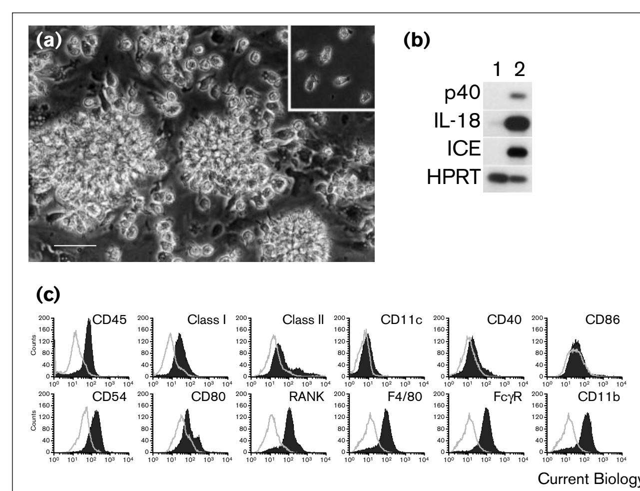
Results and discussion

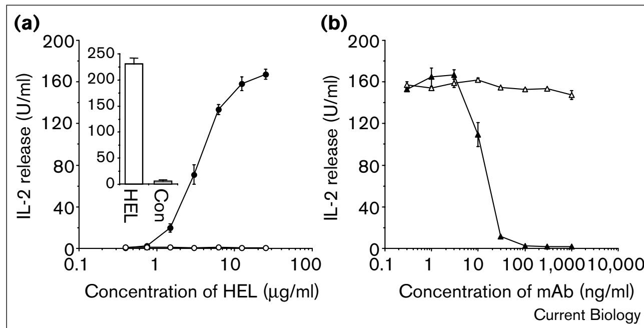
ESF116 is a novel ES cell line derived from the epiblast of a delayed-implanting CBA/Ca blastocyst [7] and is karyotypically male and germ-line competent. This line could be maintained undifferentiated on mouse embryonic fibroblasts but produced embryoid bodies (EBs) when grown in suspension. After culture for 14 days, EBs were plated onto tissue culture plastic in medium supplemented with growth factors implicated in the differentiation of DCs. These conditions promoted the adherence of EBs and the outgrowth of a variety of differentiated cell types. Of all cytokines tested (see Supplementary material), the combination of granulocyte/macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-3 uniquely supported the development of cells with an appearance suggestive of DCs within 4 days of culture. These cells expanded rapidly over the ensuing 7–10 days to form lightly adherent clusters (Figure 1a), highly reminiscent of those observed in cultures of immature DCs derived from bone marrow (bmDCs). Cells released from these clusters seeded uncolonised areas of the dish and are referred to here as ‘emigrants’. These cells displayed typical dendritic morphology (Figure 1a, inset) and formed

Figure 1



Characterisation of emigrants derived from ESF116. **(a)** Phase contrast micrograph showing the development of clusters of cells, reminiscent of cultures of bmDCs. Inset, emigrants released from clusters showing characteristic dendritic morphology, including dendrites and veils of cytoplasm. The scale bar represents 50 μm. **(b)** RT–PCR analysis of RNA from ESF116 (lane 1) and emigrants derived from them (lane 2), showing upregulation of IL-12 p40, IL-18 and ICE upon differentiation. Methods used for the analysis of RNA by RT–PCR, and details of the primers used, have been described previously [15]. Expression of hypoxanthine-guanine phosphoribosyl transferase (HPRT) was used as a control. **(c)** Surface phenotype of emigrants. Filled histograms, levels of expression of the markers indicated; open histograms, background staining with irrelevant species- and isotype-matched monoclonal antibodies.

Figure 2



Antigen processing and presentation. **(a)** Processing of HEL by live (filled circles) or fixed (open circles) emigrants measured as a function of IL-2 release by 2G7.1. The inset shows the specificity of the response to HEL by comparison with an irrelevant antigen, conalbumin (con). **(b)** Inhibition of antigen presentation by emigrants upon addition of the monoclonal antibody 17-3-3S, which is specific for H-2E^k (filled triangles), but not UPC-10, an irrelevant control monoclonal antibody (open triangles). Assays of antigen processing and presentation were performed as described previously [16].

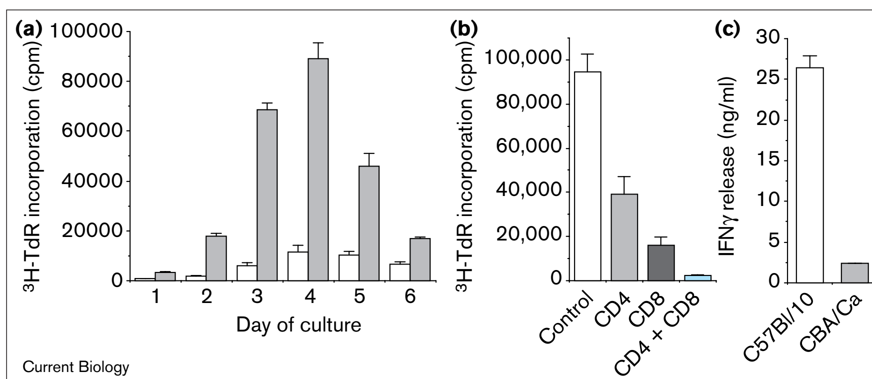
long-term cultures capable of regenerating rapidly upon routine harvesting.

To characterise these cells, we purified RNA from the ES cell line and from highly enriched populations of emigrants derived from them. Although ESF116 consistently proved negative, reverse transcription (RT)–PCR analysis of emigrants revealed the presence of mRNA for the p40 subunit of IL-12, IL-18 and IL-1β converting enzyme (ICE), required for the processing of pro-IL-18 to its bioactive form (Figure 1b). These findings are suggestive of an immunostimulatory function, both cytokines being actively secreted by DCs [8,9] and known to be responsible for biasing responding T cells towards a T helper 1 (Th1) phenotype. Consistent with this possibility, these cells expressed CD45, confirming their hematopoietic origin, together with class I and II MHC determinants (Figure 1c), the latter being found at low-to-intermediate levels on the cell surface because of their retention within intracellular compartments (data not shown). In contrast, these cells were negative for CD11c and the co-stimulatory molecules CD40 and CD86, but consistently expressed moderate levels of CD54, CD80 and RANK. Although they failed to express lineage-specific markers for T cells, B cells and neutrophils (data not shown), this population routinely expressed F4/80, FcγRII/III and CD11b, a phenotype shared by immature bmDCs and macrophages. Significantly, however, replating of these cells in macrophage colony stimulating factor (M-CSF) failed to promote proliferation or changes in their morphology and phenotype, suggesting that they may already have diverged from macrophage precursors. To investigate their possible commitment to the DC lineage, we therefore assessed their propensity for antigen processing and their ability to stimulate primary T-cell responses.

The ability of emigrants to process foreign antigen was measured as a function of their ability to stimulate 2G7.1, a T-cell hybridoma specific for hen egg lysozyme (HEL) in the context of H-2E^k (Figure 2a, inset). Figure 2a shows the dose-dependent release of IL-2 by 2G7.1 cultured in the presence of live cells pulsed with whole HEL, but their failure to respond following prior fixation of emigrants with paraformaldehyde to prevent endocytosis. Furthermore, presentation of HEL was entirely inhibited upon addition of a monoclonal antibody specific for H-2E^k, but not a control monoclonal antibody (Figure 2b), confirming presentation of the antigen in a classic, MHC-restricted fashion.

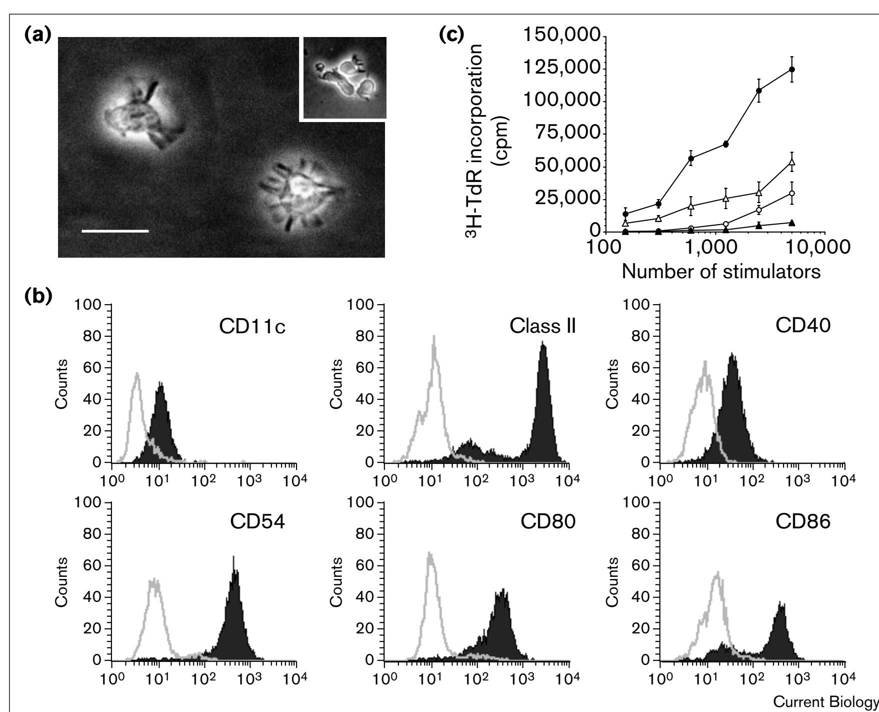
We next investigated the ability of emigrants to stimulate primary T-cell responses in the mixed leukocyte reaction (MLR). Mitomycin C-treated stimulators were cultured with purified T cells from either syngeneic or allogeneic mice, and proliferative responses measured at 24 hour intervals. Figure 3a shows significant proliferation of naïve

Figure 3



Immunostimulatory capacity of emigrants. **(a)** Kinetics of the proliferative response of naïve allogeneic (C57Bl/10; grey bars) and syngeneic T cells (CBA/Ca; white bars) upon co-culture with emigrants. **(b)** Inhibition of the proliferative response on day 4 of culture by blocking monoclonal antibody to CD4 (YTS177) and/or CD8 (YTS105). **(c)** Release of IFN-γ in co-cultures of emigrants with either syngeneic or allogeneic T cells, measured in a standard sandwich enzyme-linked immunosorbent assay (ELISA).

Figure 4



Maturation of esDCs. **(a)** High-power phase-contrast micrograph of esDCs cultured overnight in LPS, showing acquisition of many dendrites and veils of cytoplasm. The inset shows untreated esDCs for comparison. The scale bar represents 10 μm. **(b)** Flow cytometric analysis of mature esDCs (filled histograms). Open histograms represent the levels of background staining obtained with species- and isotype-matched monoclonal antibodies. **(c)** Enhanced stimulation of the allogeneic MLR by mature esDCs (filled circles) compared with immature emigrants (open circles) measured on days 2–3 of the culture period, prior to the acquisition of significant immunostimulatory activity by untreated esDCs (see Figure 3a). Syngeneic responses elicited by mature esDCs (open triangles) and immature emigrants (filled triangles) are included for comparison.

allogeneic T cells, compared with syngeneic controls, the response peaking on day 4 of culture. Significantly, proliferative responses were partially inhibited by non-depleting CD4 and CD8 monoclonal antibodies when added singly, and were completely abrogated by a combination of the two (Figure 3b), suggesting that both class I-restricted cytotoxic T lymphocytes (CTL) and class II-restricted Th cells actively contribute to this response. These findings unequivocally identify emigrants as belonging to the DC lineage: consequently, these cells have been designated ES cell-derived DCs (esDCs) to reflect their lineage commitment.

In accordance with the results of RT–PCR analysis (Figure 1b), supernatants from cultures containing allogeneic, but not syngeneic, T cells contained 500–700 pg/ml bioactive IL-12, while up to 1200 pg/ml IL-18 could be detected throughout, suggesting its constitutive secretion. As both cytokines contribute to the polarisation of responding cells towards a Th1 phenotype, we measured the release of interferon-γ (IFN-γ) and IL-4. As anticipated, high levels of IFN-γ were evident in supernatants from allogeneic cultures, whereas syngeneic T cells released significantly less (Figure 3c). IL-4, by contrast, could not be detected in either setting (data not shown). Although the stimulation of a potent Th1 response is reminiscent of the lymphoid-related subset of murine DCs [10], the absolute dependence of esDCs on GM-CSF and their lack of expression of CD8α and Dec-205 (data not shown) strongly favour a myeloid origin. These findings add weight to suggestions that the ability to elicit a Th1 response is not a property confined to particular DC subsets, but is influenced, instead, by environmental factors [11].

To determine whether esDCs might be induced to mature in a co-ordinated fashion, cells were harvested and replated for 3 days, 1 μg/ml lipopolysaccharide (LPS) being added for the final 24 hours. Following exposure to LPS, these cells acquired a highly dendritic appearance (Figure 4a), with more prominent veils of cytoplasm than their immature counterparts (Figure 4a, inset). Flow cytometry revealed the appearance of the DC-restricted marker CD11c and upregulation of MHC class II and the co-stimulatory molecules CD40, CD54, CD80 and CD86 (Figure 4b). Consistent with their phenotype, mature esDCs elicited greatly enhanced responses among allogeneic T cells (Figure 4c), frequently stimulating proliferation above background levels at densities of 300 cells per well, such responses peaking 24–36 hours earlier than those stimulated by their immature counterparts.

The derivation of long-term cultures of DCs from pluripotent ES cells has implications for our understanding of DC ontogeny and function. The requirement for IL-3 to secure the development of esDCs from EBs, but the absence of IL-3Rα chain expression by the resulting emigrants (data not shown), strongly suggests esDC development from an early IL-3-dependent hematopoietic progenitor, similar to

that described in humans [12]. Significantly, the lack of requirement for exogenous IL-3 for the development of DCs from bone marrow suggests that esDCs represent a population more primitive than previously described, a possibility supported by the lack of spontaneous maturation commonly observed among bmDCs: esDCs maintained continuously in culture for 4 weeks without harvesting showed no detectable changes in surface phenotype or stimulatory capacity. The evident stability of their phenotype over time, but their ability, nevertheless, to respond to maturation stimuli, make this novel source of DC particularly amenable to exploitation experimentally, as does their potential to express heterologous genes introduced at the ES cell stage. Indeed, the development of protocols for the production of ES cells in which both alleles of a target gene have been disrupted [13] raises prospects for the production of stable lines of 'knockout' DCs for elucidating gene function. Furthermore, the recent description of pluripotent ES cells derived from human blastocysts [14], holds promise for the application of our approach to the identification of novel targets for immune intervention in human disease.

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Supplementary material

Supplementary material including additional methodological detail and data implicating GM-CSF in the expansion of esDCs is available at http://current-biology.com/supmat/supmatin.htm.

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