In vitro hematopoiesis produces a distinct class of immature dendritic cells from spleen progenitors with limited T cell stimulation capacity

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Abstract

The study of dendritic cells (DC) has been hampered by the difficulty of isolating rare cells for analysis of their phenotype and function. Interpretation of the DC lineage has been largely influenced by studies on cell populations which can be readily isolated and amplified in the presence of cytokines. Long term cultures (LTC) from murine spleen have been shown to support continuous in vitro hematopoiesis of DC dependent on interaction with a stromal cell monolayer. LTC-DC represent a single, stable class of DC derived by constant turnover of spleen DC progenitors maintained within stroma. They represent a resident DC population in spleen. The functional characteristics of LTC-DC have been studied in terms of capacity to stimulate T cells and response to activation by environmental stimuli. LTC-DC have many morphological, phenotypic and functional properties reflecting an immature or partially mature, marginal zone-like CD4⁻CD8⁻ splenic DC subset. They are highly endocytic and can process and present protein antigen to naive hen egg lysozyme (HEL)-specific MHC-II-restricted TCR-Tg CD4⁺ T cells. They do not, however, induce T cell proliferation in a mixed lymphocyte reaction. LTC-DC do not respond in a typical fashion to common DC activators like LPS and CD40L. They upregulate MHC-I and CD80/CD86 but not MHC-II and CD40. They reflect an endogenous, immature DC subset in spleen with properties distinct from immature DC located in peripheral tissues.

Introduction

Many subsets of dendritic cells (DC) have been identified which differ in phenotype and function. Over time these subsets have been classified variably in terms of phenotype, tissue-specific origin and state of activation or maturation (1). The generally accepted model is that DC located in peripheral tissues are immature and that after exposure to antigen in the presence of inflammatory factors these cells mature and migrate to lymphoid organs where they present antigen for T cell stimulation. This model has been well supported by studies on DC which capture antigen in sites such as skin, lung and gut (2–4). Migration of antigen-carrying DC from peripheral sites of infection into draining lymph nodes is associated with maturation of DC and is a key event in the priming of the immune response (5). DC maturation occurs upon exposure to microbes or inflammatory factors. Mature DC show upregulation of cell surface markers like CD80/CD86, MHC-II and CD40 and capacity to activate naive T cells. In contrast, immature DC are thought to play an important role in tolerance induction (6,7). Evidence that immature DC subsets are also located within lymphoid tissues such as spleen and lymph nodes (8) supports an important role for DC in the maintenance of self tolerance. Immature DC are characterized by high endocytic capacity and poor capacity to process and present antigen to T cells. DC in the steady-state are expected to be immature and play an important role in sampling the environment and maintaining tolerance.

Attempts to classify the DC subsets in different tissues are continuing with a view to solving the puzzle of DC development. However, the isolation of fresh murine DC is tedious, involving large amounts of tissue and labour intensive isolation procedures (9). This can result in significant leukocyte impurities and unintentional removal of some DC subsets (10). As a result, the classification of DC into different subsets and states of maturation is a very difficult task. Many in vitro...
protocols have been developed to propagate DC from progenitors using cocktails of cytokines. In mice, this involves isolation of progenitors from organs like bone marrow, blood and liver, and culture of cells with combinations of factors like granulocyte macrophage colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α) and IL-4 (11). Adoption of these in vitro cultures has led to significant advances in DC biology (12). However, addition of growth factors like TNF-α as well as the process of culturing cells in vitro can induce activation of DC with loss of their capacity to endocytose antigen for T cell stimulation (13). In vitro activation of DC can complicate the functional and phenotypic identification of DC subsets within tissue sites.

More recently, dendritic-like cells have been generated from precursor cells without the need for added cytokines, provided they are cultured in the presence of stromal cell monolayers containing endothelial cells. In one in vitro model, endothelial cells supported the development of DC from blood-derived monocytes (14). Human umbilical vein endothelial monolayers supported the differentiation of DC from blood monocytes within 2 days of culture by a process involving trans-endothelial migration (14). Long term cultures (LTC) established from mouse spleen in the absence of exogenous cytokines are a proven method for generating large numbers of DC where hematopoiesis is supported by stromal cell monolayers comprising endothelial and fibroblastic cells (15–18). LTC support the continuous differentiation of spleen progenitors into non-adherent cells displaying characteristics common to DC (15–18). The production of DC in LTC has been confirmed by preparation of subtracted libraries representing precursor and progeny cells produced in LTC. Sequencing of differentially expressed clones indicated that cell differentiation within LTC was marked by upregulation of genes common to DC (19). Multiple LTC have been shown to reproducibly generate DC independently of GM-CSF (20). LTC represent a niche environment for reproducible hematopoiesis of spleen DC from progenitors contained within a stromal cell monolayer (17,18). The ease with which cells are produced in LTC would suggest that DC generated in LTC reflect the normal in situ production of a common class of DC in spleen.

Lymphoid organs are known to contain a major population of immature DC that play an important homoeostatic role in tolerance. Mature DC in these organs are thought to enter from peripheral sites carrying antigen for T cell activation (8,21). The majority of spleen DC in both mouse and human have been shown to represent immature DC (8). Spleen is known to contain three subpopulations of DC identifiable on the basis of the expression of the CD4 and CD8 markers: CD4<sup>+</sup>CD8<sup>−</sup> DC, CD4<sup>−</sup>CD8<sup>+</sup> (CD8<sup>+</sup>) DC and CD4<sup>+</sup>CD8<sup>+</sup> (CD4<sup>+</sup>) DC (22). The CD8<sup>+</sup> DC subset which specifically expresses CD205 is thought to represent a class of DC located in T cell areas (23), while the CD4<sup>+</sup>CD8<sup>−</sup> reside in the marginal zone (24). This study is concerned with spleen-derived DC produced in LTC and their functional characterization in relation to known DC subsets in spleen. It demonstrates that LTC-DC are representative of a functionally distinct endogenously produced immature DC population resident in spleen which is highly endocytic, refractory to activation by common DC activators and has only weak capacity to activate T cells.

**Methods**

**Cell isolation from mice**

Inbred mice were bred in the John Curtin School of Medical Research (Australian National University, Canberra, Australia) under specific pathogen-free conditions. BALB/c, B10.A(2R) and C57BL/6J male and female mice aged between 5 and 15 weeks were used throughout this study. TCR-transgenic (TCR-Tg) mice expressing TCR α and β chains from the 3A9 T cell hybridoma specific for hen egg lysozyme (HEL) peptide presented by I-A<sup>+</sup> (25) and bred on to the CBA/H (H-2<sup>b</sup>) background were kindly provided by Chris Goodnow (Australian National University). Mice were housed and handled according to the guidelines of the ANU Animal Experimentation Ethics Committee. Spleens or lymph nodes were harvested from mice euthanized by cervical dislocation. Single cell suspensions were prepared by forcing tissue through a fine wire mesh using a syringe plunger followed by repeated pipetting in culture medium. RBC depletion involved cell lysis in 5 ml lysing buffer [0.14 M NH₄Cl, 0.017 M Tris-base (pH 7.5)] for 5 min at 20°C followed by three washes in ice-cold medium.

**In vitro generation of DC in spleen LTC**

Primary spleen LTC were established as described previously (15–18) in supplemented DMEM (Gibco BRL, Grand Island, NY) containing 10% fetal calf serum (sDMEM) as detailed previously (18). After several weeks of culture with medium change as needed, LTC were selected which contained a stromal cell monolayer of fibroblasts and endothelial cells that continually supported the proliferation and differentiation of progenitors into non-adherent dendritic-like cells. LTC sublines were generated by dissociating stroma in LTC using plastic scrapers and transferring aliquots of these together with non-adherent cells into new tissue culture flasks. Cultures were easily maintained with medium change every 2–3 days. LTC generated as many as 1 × 10<sup>6</sup> non-adherent cells per 75 cm<sup>2</sup> of stroma every 2 days. Non-adherent cells were collected at medium change for analysis. Cells produced in LTC have been extensively characterized as DC (15–20,26). Results presented here are representative of many replicate analyses on DC produced by multiple LTC derived from two different strains of mice.

For treatment, LTC-DC were cultured for 24 h without stroma in medium alone, or in the presence of various agents. Treatments included 10 µg/ml LPS (Sigma, MO, USA) and/or 1/100 dilution of CD40L titrated to produce maximal B cell proliferation. CD40L was prepared as the supernatant of paraformaldehyde-fixed CD40L-baculovirus-infected SF9 cells, kindly provided by Virginia McPhun (Australian National University). For antigen pulsing, LTC-DC (10<sup>6</sup>) were cultured without stroma for 24 h in the presence of 10 µg/ml HEL (Sigma) with or without 10 µg/ml LPS. Cells were washed twice in PBS (pH 7.4) to remove soluble protein antigen before addition to cultures of purified T cells.
Resin-embedded sections of non-adherent cells from LTC were prepared for transmission electron microscopy (TEM). Cells (10^7) were fixed overnight in 2% gluteraldehyde/0.1 M sodium cacodylate buffer (pH 7.4), washed in sodium cacodylate buffer (pH 7.4), stained in 1% osmium tetroxide for 1.5 h, repeatedly washed in ddH2O and stained in 2% uranyl acetate for 2 h. They were then washed in ddH2O and dehydrated in increasing concentrations of acetone. Cells were infiltrated with Spurr’s resin and cut into thin (80 nm) sections using a diatome diamond cutter followed by staining with lead citrate (2.6% lead nitrate, 3.5% sodium citrate, 0.16 M sodium hydroxide) for 15 min. After washing with ddH2O, sections were air-dried and viewed in a Hitachi 7000 transmission electron microscope (Hitachi, Japan) at 60 Kv.

Endocytosis assay

LTC-DC were assessed for capacity to endocytose FITC-OVA (Molecular Probes). Cells were washed and placed on ice for 10 min before addition of 100 μg/ml OVA-FITC in a total volume of 100 μl sDMEM. Cells were then incubated for 45 min at 37°C. Control cells were kept on ice for 45 min. Endocytosis was halted by addition of 100 μl of ice-cold PBS/0.1% NaN3. Cells were washed three times and resuspended in PBS/0.1% NaN3 for analysis of fluorescence uptake by flow cytometry.

Preparation of purified CD4+ T cells from 3A9 TCR-Tg mice

A purified population of CFSE labelled CD4+ T cells was prepared from 3A9 TCR-Tg mice. A single cell suspension of spleen cells was prepared. Pelleted cells (<10^6/ml) were labelled by resuspension in 1 ml sDMEM and addition of CFSE while vortexing to give a final concentration of 5 μM. Cells were then incubated at room temperature for 5 min before RBC depletion. CD4+ T cells were then isolated by depletion of unwanted cell subsets using magnetic Dynabeads coupled to sheep Ig anti-rat Ig (Dynal, Oslo, Norway). The procedure involved labelling unwanted cells with the following combination of rat antibodies: B220 (RA3-6B2), CD8α (53-6.7), CD11b (M1/70), MHC-II (TIB120), DEC-205 (NLDC-145), DC (3D101), macrophages (F4/80). Specific antibody was absorbed to cells for 30 min on ice. Cells were then washed three times in ice-cold sDMEM by centrifugation. Sheep Ig anti-rat Ig Dynabeads were added to cells for 30 min at 4°C with rotation using a bead to cell ratio of 4:1. Bead-coated cells were exposed to a magnet for 2 min, allowing aspiration of CD4+ T cells in solution. Purification typically resulted in >90% purity of cells as assessed by antibody staining and flow cytometry.

Mixed lymphocyte reactions

To assess lymphocyte proliferation through DNA synthesis, RBC-depleted spleen cells were cultured in triplicate at 2 × 10^5 cells/well in 96-well plates together with diluting concentrations of LTC-DC as stimulator cells in a total volume of 200 μl sDMEM. Concanavalin A was used as a control stimulator of lymphocytes. Prior to culture, LTC-DC were irradiated (20 Gy on a Cobalt60 source; Commonwealth Scientific and Industrial Research Organisation [CSIRO], Black Mountain, ACT, Australia). DNA synthesis was assessed over the last 16 h of a 72 h culture through addition of 1 μCi/well of ³H-thymidine (³H-T) (Amersham, Buckinghamshire, UK). Cells were harvested and measured using CellQuest software (Becton Dickinson) and involved post-acquisition gating to obtain information on cell subsets. For analysis of lymphocytes amongst a mixed leukocyte population, cells were gated out on the basis of FSC and side scatter (SSC) properties. Lymphocyte blastogenesis was assessed on the basis of FSC above background and cell division by dilution of the intracellular dye, 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, OR, USA). In some experiments, 5 μl of 100 μg/ml propidium iodide (PI) was added to 0.2 ml cells in PBS prior to flow cytometry for discrimination of dead cells. For total cell counts, 1000 CaliBRITE beads (Becton Dickinson) were added to 200 μl of cell solution for estimation of total cell number in solution based on ratio of cell subsets to beads in FSC and SSC analysis of collected data.
were harvested on to glass fibre filters then saturated in MicroScint scintillation fluid (Packard, CT, USA) for measurement of label incorporation in a Top-Count scintillation counter (Packard). Responses were reported as mean c.p.m. ± SE of the mean of triplicate samples. Controls included responders or stimulators alone. Background ³H-T incorporation due to stimulators alone was <500 c.p.m.

Results

Morphology and ultrastructure of DC produced in LTC

Non-adherent cells produced in LTC were predominately spherical, internally complex and displayed dendrites visible under light microscopy (Fig. 1a and b). These cell populations and their supporting monolayer of stromal cells have maintained similar morphological characteristics over the course of this and previous studies (15,18). LTC represent a highly reproducible culture system for production of a population of DC of consistent morphology, phenotype and function.

Non-adherent cells produced in LTC can be characterized as DC on the basis of their unique morphology and ultrastructure using TEM. Most cells were large with an irregular outline and an average diameter of 20 µm (Fig. 1c). Cell nuclei were predominantly irregular, eccentric and spherical or kidney-shaped, containing a peripheral rim of chromatin. The majority of cells displayed high cytoplasmic to nuclear ratio, with many mitochondria and abundant endosomes of varying density (Fig. 1d). Non-adherent cells produced in LTC have morphology and ultrastructure consistent with a metabolically active, endocytic, dendritic-bodied cell, analogous to previously described DC (28).

Phenotype of DC produced in LTC

In the absence of a definitive cell surface marker, DC are discriminated from other leukocytes through expression of multiple markers (29). Markers expressed by LTC non-adherent cells were analysed by labelling cells with specific antibodies and measurement of antibody binding by flow cytometry. Light scatter properties revealed that the majority of LTC non-adherent cells displayed high FSC consistent with their large size and high SSC consistent with cellular complexity. PI staining of cells showed that LTC maintain a population of ~25% dead cells reflecting the turnover of cells in a continuous culture system.

Antibody staining and flow cytometry were used to assess surface marker expression after gating to exclude dead cells and cell debris (Fig. 2). Antibody activity was titrated initially on positively staining control cell populations isolated from spleen.
Non-adherent cells produced in LTC expressed high levels of CD11c, a common marker on DC (9). Some cells expressed low levels of DEC-205, a marker associated with DC present in T cell areas of spleen (30). Very few LTC cells expressed the molecule recognized by the 33D1 antibody, thought to be expressed at low levels by marginal zone DC (24). Markers for lymphoid cells including Thy1.2, B220, CD8α and CD4 were not expressed. Cells did however express high levels of the myeloid marker CD11b, but did not express the macrophage/myeloid marker F4/80. Cells expressed moderate levels of MHC-I and CD80 but the expression of CD40 and CD40L was low or undetectable. Cells expressed high levels of CD86 typical of mature DC (29) but high levels of FcγRII/IIIR typical of immature DC. This staining pattern is typical of immature DC (9,31) except for the high expression of CD86. The majority of cells did not express MHC-II, but a subpopulation (6.9%) of LTC cells expressed high levels of MHC-II characteristic of mature DC (9). There was almost no expression of CD34, a marker of hemopoietic stem cells. If stem cells are produced in LTC, they are in very low numbers or contained within the stromal cell layer (26).

An important characteristic of immature DC is their capacity to take up antigen through endocytosis. This property is thought to enable DC to sample their surroundings for potential pathogens, but is rapidly down-regulated upon cell maturation (12). Non-adherent cells produced in LTC were incubated with OVA-FITC and monitored for fluorescent uptake by flow cytometry after incubation at 37°C versus 0°C. At 37°C, cells were able to efficiently take up soluble OVA-FITC. Greater than 95% of cells absorbed the protein after 45 min of culture compared with ~0.5% at 0°C, a temperature which inhibits endocytic ability (Fig. 2).

The majority of non-adherent cells present in LTC display many properties consistent with a predominately immature DC characterized by high endocytic capacity, high expression of FcγRII/IIIR and low expression of the molecules MHC-II, CD40 and CD40L. Most cells are apparently immature yet express high levels of the costimulatory molecule CD86 common to mature DC. A small subset of mature MHC-II+ cells was also detected.

**Responsiveness of LTC-DC to activation**

One question addressed was whether non-adherent cells produced in LTC respond to factors commonly known to activate DC. After exposure to LPS and CD40L, subsets of immature DC have been shown to undergo functional maturation, rapidly downregulating receptors for endocytosis and reducing their endocytic capacity (12). They also upregulate expression of immunostimulatory molecules such as MHC and CD80/86, important for antigen presentation and lymphocyte activation.

LTC-DC were collected and recultured on the plastic surface of tissue culture flasks in the absence of stroma. Cells were cultured for 24 h either in medium alone or in medium supplemented with activators. Cells were then collected and assessed for marker expression and endocytic capacity in comparison with cells freshly isolated from a stroma-dependent LTC. Activation conditions included reculture in the absence of stroma on plastic tissue culture flasks alone, or with added LPS or CD40L or both LPS and CD40L. Each of these conditions is a known potent activator of DC (13,31).

Non-adherent cells produced in LTC upregulated MHC-I and CD86 expression upon reculture in medium alone (Fig. 3a). This result suggested that the stromal environment of LTC did not lead to activation of developing DC. In fact, when cells were collected and cultured in the presence of known DC activators like LPS and CD40L, or a combination of...
these two agents, there was no further upregulation of marker expression upon reculture, indicating no further activation of cells. Only slight upregulation of CD40 and CD40L was detected and again these effects were not dramatically enhanced by addition of LPS or CD40L (Fig. 3a). Upregulation of CD40L could be a result of soluble CD40L adhering to LTC cells in culture even though cells were extensively washed prior to staining. Each of the treatments led to partial but very weak downregulation of endocytic capacity and of FcγRII/IIIR expression. This is also known to occur during the process of DC maturation (29).

The changes induced by LPS were found to vary slightly for non-adherent cells produced in different LTC. In the majority of cases, there was no upregulation in MHC-II expression. However, on three separate occasions, LPS was found to induce a slight upregulation of MHC-II above that induced by reculture of cells in medium alone (see Fig. 3b). This was also associated with noticeable upregulation of MHC-I and CD86 on non-adherent cells collected from these cultures.

LTC-DC can be induced to modulate marker expression and endocytic capacity, including upregulation of MHC and CD86 together with downregulation of FcγRII/IIIR and endocytic capacity. However, these effects were only minor, with treated cells still maintaining high endocytic capacity, high FcγRII/IIIR expression and weak expression of CD80, CD40 and CD40L and weak or no expression of MHC-II. Furthermore, treatment with TNF-α did not induce phenotypic changes in LTC cells (data not shown). In general, non-adherent cells derived from LTC are relatively refractory to common DC activators although they do show some maturation in response to treatment with activating agents. The in vitro production of DC in stroma-dependent LTC gives rise to immature DC most of which are not activated by the culture procedure.

Capacity to stimulate antigen-specific naive T cells

One important characteristic of immature DC is their capacity to take up and process antigen for presentation and subsequent activation of antigen-specific naive T cells. This effect...
can be dramatically enhanced by prior maturation of DC (9,33). The lymphostimulatory capacity of LTC-DC has not previously been shown to include MHC-restricted antigen-specific activation of naive T cells. With the relatively low level of MHC-II expression on the majority of LTC-DC, it was unclear whether cells could process and present protein antigen in order to stimulate naive CD4+ T cells. Since a minor subpopulation (6.9%) of LTC cells is shown to express high levels of MHC-II along with CD86 (Fig. 2), it is possible that LTC can develop into DC with immunostimulatory capacity within the LTC environment. The addition of LPS as a DC activator would be expected to upregulate immunostimulatory markers on cells and so increase the T cell response generated.

Non-adherent cells were collected from LTC and pulsed with HEL protein. Cells were then tested for their ability to stimulate purified CD4+ T cells from 3A9 TCR-Tg mice. Purified CD4+ T cells display the TCR derived from the 3A9 hybridoma specific for a HEL peptide presented in the context of IAk (25). T cells were purified from the spleen of TCR-Tg mice by antibody-mediated negative depletion to remove unwanted cell types. This protocol was chosen to avoid stimulation of T cells which occurs in positive selection methods. The purification method involved removal of MHC-II+ APC including DC, to preclude induction of T cell responses by endogenous APC. The depleted cell population showed enrichment for T cells having ~90% CD3+ cells and ~1% MHC-II+ cells (Fig. 4a). All gated CD4+ T cells expressed the TCR-Vβ8.2 epitope of the transgene (Fig. 4a). The MHC-specificity of the response was tested by comparing responses generated by HEL-pulsed DC collected from LTC established from B10.A(2R) (IAk) mice and from C57BL/6J (IAβ) mice. The inclusion of control IAβ-positive

![Diagram](image_url)

**Fig. 4.** LTC-DC stimulate antigen-specific naive CD4+ T cells from TCR-Tg mice. Non-adherent cells collected from LTC were assessed for capacity to stimulate purified CD4+ T cells from 3A9 TCR-Tg mice. (a) CD4+ T cells were enriched from lymph node and spleen by labelling cells with rat antibodies specific for MHC-II (IAk: TIB 120), B220 (RA3-6B2), macrophages (F4/80), CD11b (M170), DC (33D1), DC (DEC-205) and CD8α (53±6.72) and magnetic removal of unwanted cells using sheep anti-rat Ig Dynabeads. The resulting population was highly enriched for CD3+ T cells and mostly depleted of MHC-II+ APC. This was confirmed by staining with the AF6 120.1 anti-MHC-II antibody which recognizes a different epitope to TIB 120 used in the T cell purification procedure. CD4+ T cells expressed the transgene product TCR-Vβ8.2, as detected by binding of specific antibody (F23.2). (b) LTC-DC were pulsed with HEL protein (10 μg/ml) in the presence and absence of LPS (10 μg/ml) for 24 h. Cells were washed and 10^5 cells were assessed for capacity to stimulate T cells for 3 days after culture. PI (25 μg/ml) was included to identify viable CFSE-labelled cells. Specificity of antibody labelling was monitored by background binding of isotype control antibody or medium alone (Nil). Numbers shown represent cell percentages in gated sectors. *Indicates MFI change in CD3e expression by CD69+ cells relative to CD69− cells in the same plot.
stimulators also controlled for stimulation of T cells by any residual HEL taken up and presented by residual endogenous APC. Some cells were also treated with LPS at the time of antigen pulsing to determine the effect of LPS in activating LTC-DC to be more immunostimulatory.

In order to measure T cell responsiveness, TCR-Tg CD4+ T cells were examined by flow cytometry for upregulation of expression of the early activation marker CD69, downregulation of CD3ε (34), blastogenesis assessed as an increase in FSC and proliferation assessed by reduction in the mean fluorescence intensity (MFI) of the intracellular dye, CFSE. Measurement was performed at 0.5 and 3 days after coculture with HEL-pulsed LTC cells or medium alone as a control. Naive HEL peptide-specific TCR-Tg T cells responded well to HEL-pulsed IAk-positive LTC cells. After 0.5 days, 27% of CD4+ T cells responded by upregulating CD69 expression above background (Fig. 4b). CD3ε expression was also reduced on CD69+ cells relative to CD69- cells with a reduction in MFI of 70 (Fig. 4b). At this early stage, 7.9% of cells showed signs of blastogenesis relative to controls. By 3 days, 40% of cells showed upregulation of CD69 with 51% of viable T cells (assessed by PI exclusion) showing blastogenesis. Of these T cell blasts, most had undergone at least one cell division and up to a maximum of four divisions as determined by reduction in CFSE intensity (Fig. 4b). T cell responses induced by IAε-positive LTC-DC were minor, showing only a 10% upregulation in CD69 expression at 3 days, blastogenesis in only 10% of cells and no cell proliferation indicated by CFSE staining. This confirmed an MHC-restricted response with no evidence for antigen presentation by endogenous APC present in the enriched T cell population.

LPS treatment of LTC-DC following antigen pulsing of LTC-DC had very little effect on T cell response (Fig. 4b). There was, however, early indication by 0.5 days of an increased T cell response following treatment of IAε-positive DC with LPS. This was indicated by a 17% increase in CD69 expression at 0.5 days which dropped to a 6% increase after 3 days. There was also evidence of greater reduction in the MFI of CD3ε on activated T cells. The effect of LPS treatment of LTC-DC on T cell responsiveness was noticeable at 0.5 days but was reduced by 3 days after stimulation. LTC-DC have capacity to take up and process proteins into peptides for presentation on MHC-II and to stimulate activation and proliferation of antigen-specific naive CD4+ T cells. The activating effects of LPS on LTC-DC were noticeable but weak, which is consistent with the minor effects of LPS on the expression of CD86 and MHC-II seen in Fig. 3.

Inability of LTC-DC to stimulate an MLR

Consistent with their capacity to stimulate naive TCR-Tg CD4+ T cells, previous investigations have demonstrated that all tested LTC produce cells which can process and present conalbumin to the Th2 D10.G4.1 helper cell line (35). Despite this, LTC-DC derived from several cultures of B10.A(2R) mice were found to be incapable of stimulating an MLR in either allogeneic BALB/c mice or syngeneic C57BL/6J mice (Fig. 5). Responder lymphocytes from both strains could, however, respond to stimulation with concanavalin A.

Discussion

This study has revealed that LTC generate non-adherent cells with unique DC properties. Cells display many of the structural, phenotypic and functional properties commonly used to define DC. Short cytoplasmic extensions, irregular nuclei with a peripheral rim of chromatin and high mitochondrial and endosomal content characterize LTC-DC and are known characteristics of DC (28), particularly those in an immature state (12). LTC-DC have a CD11c+ CD11b+ DEC-205- CD40- CD4- CD8α- phenotype. They also have high
expression of FcγII/IIIIR, low expression of MHC-II, CD80 and CD86 and capacity to stimulate lymphocytes, including MHC-I, MHC-II, CD80, CD40L and CD40. All of these markers are reportedly weakly expressed on immature DC. LTC-DC have high expression of FcγII/IIIIR and a high capacity to endocytose protein antigen, typical of immature DC. In contrast, they have no capacity to stimulate lymphocyte proliferation in an MLR but can process protein antigen for presentation on MHC-II for stimulation of antigen-specific TCR-Tg T cells, a property of mature DC. A partially mature phenotype for LTC-DC is consistent with high endocytic capacity and limited T cell activation capacity. Non-adherent LTC cells consistently express high levels of CD86 but low to very low levels of other immunostimulatory markers like MHC-I, MHC-II, CD80, CD40L and CD40. All of these markers are reportedly weakly expressed on immature DC but are upregulated upon DC maturation (12,31).

Despite their immature characteristics, LTC-DC are capable of stimulating antigen-specific responses from T cell clones and TCR-Tg T cells. Previously it was shown that DC in a range of maturation states including immature DC derived from bone marrow could process and present antigen to T cells (38). It is not clear, however, why LTC-DC cannot stimulate T cells in an allogeneic MLR, one of the first functional features identified in DC (39). Lack of T cell responses in an MLR might be a consequence of low expression of MHC-II on most LTC-DC, coupled with differences in activation thresholds of the responding T cell populations. It could also be argued that this is due to differences in the ligand avidity of the TCR on a clonal T cell population like D10.G4.1 or TCR-Tg T cells versus T cell populations that have a diverse TCR repertoire as in an MLR. However, recent studies have demonstrated that the response in T cells generated by DC in an allogeneic MLR may not be as clear cut as direct presentation of alloantigens by the stimulating DC (40). It was demonstrated that in order for allogeneic DC to stimulate a full response in MLR, the...
presence of syngeneic DC present in the responding leukocyte population was required (40). It was shown that allogeneic DC transferred MHC-II to syngeneic DC through CSN particles of \(<200\) nm in diameter, which endowed these syngeneic DC to stimulate T cells in a MLR (40). LTC-DC may not produce the factors required for this response to occur.

While a small subset of non-adherent LTC-DC expresses high MHC-II and clearly has capacity to initiate MHC-II-restricted T cell responses in 3A9 TCR-Tg mice, the majority of cells lack expression of MHC-II even after stimulation with LPS and CD40L. This contrasts with the majority of reports on DC (12). Lack of MHC-II expression could be a result of the in vitro culture conditions used to propagate LTC-DC. Freshly isolated DC from skin have been shown to dramatically decrease MHC-II synthesis during in vitro culture, with cessation after 3 days, although MHC-II is stably expressed on the cell surface (41). LTC-DC do synthesize MHC-II components (unpublished data).

It is thought that with their high endocytic capacity, LTC-DC lose surface MHC-II in the rapid turnover of their plasma membrane. Recent evidence has shown that expression of MHC-II on the surface of DC is tightly controlled by the rate of endocytosis, with highly endocytic cells reabsorbing and degrading MHC-II/peptide complexes much faster than mature weakly endocytic DC (42).

LTC-DC have low propensity to respond to factors known to mature or activate commonly isolated subsets of DC. LPS treatment, CD40 crosslinking and TNF-\(\alpha\) treatment have limited or no influence on either marker expression or endocytic function of LTC-DC. Furthermore, while reculture of LTC cells enhanced their expression of MHC-I and CD86, other markers like MHC-II and CD80 failed to upregulate. It has been demonstrated that DC treated with IL-10 (43) or TGF-\(\beta\) (44) become less responsive to maturation signals. Analysis of gene expression has so far indicated that these two factors are not produced in LTC (unpublished data). Immature DC have also been propagated from BM with low doses of GM-CSF that are resistant to the maturational effects of LPS, CD40 ligation and TNF-\(\alpha\) treatment (45). It has been suggested that these DC may induce T cell tolerance (41). LTC-DC clearly produce proliferative responses in TCR-Tg T cells. However, whether the end result of this T cell response is immunogenic or leads to abortive T cell proliferation involved in removing T cell clones (46,47) has not yet been addressed. This could occur if LTC-DC can stimulate regulatory T (Treg) cells. The outcome of T cell stimulation in terms of polarization of the T cell response to Th1, Th2 or Treg cells is currently under investigation.

Indeed, the finding that LTC-DC are refractory to further maturation with activation stimuli, like CD40L, LPS and TNF-\(\alpha\), is consistent with a model whereby LTC perpetually produce DC to a unique point in development. In support of this is evidence that DC have been shown to have a short \(~2\) day turnover time in spleen (48) and it is possible that the majority of splenic DC would die in an immature state with very few actually becoming mature cells. The inability of LTC-DC to respond to common DC activators would also be consistent with the presence of a distinct subset of immature DC in spleen which is refractory to activating factors known to activate DC located in other anatomical sites.

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Abbreviations

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<td>CFSE</td>
<td>5-(and 6-)carboxyfluorescein diacetate succinimidyl ester</td>
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<tr>
<td>CV</td>
<td>crystal violet</td>
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<td>dendritic cells</td>
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<td>FSC</td>
<td>forward scatter</td>
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<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
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<td>LTC</td>
<td>long term cultures</td>
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<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
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<tr>
<td>SSC</td>
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<tr>
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<td>supplemented DMEM</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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References
