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Tolerance Induction
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IMMATURE DENDRITIC CELLS INDUCE HYPORESPONSIVENESS TO ALLOANTIGENS IN VITRO AND PROLONG MOUSE CARDIAC ALLOGRAFT SURVIVAL

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Running foot: Immature dendritic cells induce T cell hyporesponsiveness

With: Two Figures and 1 Table

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INTRODUCTION

The functional maturation of bone marrow (BM)-derived dendritic cells (DC) is now thought to reflect the upregulated expression of a variety of cell surface molecules that are important in antigen presentation, intercellular adhesion and T cell stimulation. These comprise major histocompatibility complex (MHC) antigens, and both intercellular adhesion (e.g. CD54 and CD58) and T cell stimulatory molecules, especially the CD28 ligands B7-1 (CD80) and B7-2 (CD86) (1,2). Although mature DC are the most potent antigen-presenting cells (APC) and inducers of naive T cell activation (3,4), there is also evidence that they play a role in central tolerance induction (5-7) within the thymus. More recently, it has been suggested that DC progenitors or functionally immature DC, that are deficient as inducers of allogeneic T cell responses, may play a role in peripheral tolerance (8-10). Moreover, the CD28 structural homologue CTLA4-Ig, a fusion protein that blocks CD28-mediated T cell stimulation inhibits T cell proliferation in allogeneic mixed leukocyte reactions (MLR) (11,12). In addition, it induces hyporesponsiveness to alloantigen in vitro (11) and donor-specific tolerance in cardiac-allografted mice (13).

Recently, we have shown that small numbers of donor-derived DC progenitors (14) can be propagated in response to GM-CSF from the BM of unmodified murine liver allograft recipients that accept their grafts spontaneously (15,16). This finding has provoked questions about the function of donor-derived, GM-CSF stimulated BM DC progenitors in relation to tolerance induction. In this study, we have investigated the

function of BM-derived DC progenitors in allogeneic systems in vitro and tested their influence on cardiac allograft rejection.

MATERIALS AND METHODS

Animals. Ten to twelve-week old C57BL/10J (B10; H-2^b, I-A^b, I-E^-) and C3H/HeJ (C3H; H-2^k, I-A^k, I-E^k) mice were obtained from The Jackson Laboratory, Bar Harbor, ME.

Propagation of bone marrow (BM)-derived DC. Bone marrow (BM) cell suspensions were prepared in RPMI-1640 (Gibco, Grand Island, NY) using conventional procedures. Two x 10^6 cells were cultured in 24-well plates in RPMI-1640 supplemented with 10% v/v fetal bovine serum (Gibco) and 500 U(2ng)/ml recombinant(r) mouse GM-CSF (Schering-Plough, Kenilworth, NJ). In some experiments, the culture medium was supplemented with r mouse IL-4 (Schering-Plough; 1000 U/ml) in addition to GM-CSF. The procedure used was modified (17) after that described by Inaba et al (18) for the propagation of large numbers of DC progenitors from normal mouse BM. The medium containing GM-CSF $\pm$ IL-4 was refreshed every two days.

Staining of cell surface antigens. Cell surface staining was analyzed by cytofluorography using a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA) as previously described (17). Staining with primary hamster or rat monoclonal antibodies (mAbs), including rat anti-B7-1 and B7-2 (PharMingen, San Diego, CA) was followed by FITC-conjugated goat anti-hamster or mouse anti-rat Igs, as described (17,19). MHC class I and class II positive cells were identified using biotin-

conjugated mouse anti-mouse mAbs (PharMingen, San Diego, CA) with FITC streptavidin (Jackson Immunoresearch Labs Inc., West Grove, PA) as the secondary reagent.

Mixed leukocyte reactions (MLR) and testing for T cell hyporesponsiveness. Splenocytes for use as responder cells, were depleted of red blood cells by lysis in Tris-buffered NH_4Cl then T-cell enriched (>95%) by sequential removal of plastic-adherent cells (1 hr at 37°C) and high affinity negative selection by passage (10 min at room temperature) through a Mouse T Cell Enrichment Column (R & D Systems, Inc., Minneapolis, MN). For MLR, 2 x 10^5 T cells were cultured with graded concentrations of γ-irradiated (20 Gy) allogeneic stimulator cells in 0.2 ml of medium for 3 days in 96-well, round-bottom tissue culture plates in 5% CO_2 in air. Alternatively, for primary MLR, 2 x 10^6 T cells were incubated with 1 x 10^6 stimulators in 24-well plates in 2 ml of medium. In some experiments, hamster anti-CD28 mAb (Pharmingen; 25μg/ml) or control hamster IgG was added to the primary MLR. After 2 days, the cells were harvested, washed in medium and depleted of Ia^+ cells with anti-I-A^b mAb (Pharmingen) plus low toxicity rabbit complement (Accurate Chemical & Scientific Corp., Westbury, NY) (17). Viable cells were isolated by centrifugation over Ficoll-Isopaque for 15 min at 350g and rested in medium for 2 days in the absence of stimulators. The pretreated T cells were then restimulated with freshly-isolated, γ-irradiated spleen cells in the absence or presence of r human IL-2 (Gibco; 50U/ml) in a secondary, 3-day MLR in 96-well round-bottom plates. For the final 18 hr, 10μl [^{3}H] TdR (1μCi) was added to each well. Cells were harvested onto glass fiber disks using a multiple cell harvester and the degree

of thymidine incorporation was determined in a liquid scintillation counter. Results were expressed as mean counts per minute (cpm) $\pm$ 1SD.

IL-2 assay. IL-2 levels in MLR culture supernatants were determined by the CTLL cell assay (20).

Heterotopic heart transplantation. The method for heterotopic heart transplantation was adapted from the rat procedure of Ono and Lindsey (21). The heart was transplanted into the abdomen with end to side anastomosis of aorta to aorta and pulmonary artery to vena cava. Rejection was defined by the cessation of cardiac contraction after daily palpation through the abdominal wall, and confirmed by histological examination. No immunosuppressive therapy was used. To test the influence of cultured cells on heart allograft survival, 2 x 10^6 cells were injected i.v. in 0.2 ml medium via the lateral tail vein, 7 days before organ transplantation.

RESULTS

Immunophenotypic analysis of GM-CSF stimulated cells. The surface immunophenotype of cells released after 8-10 days from proliferating aggregates in GM-CSF stimulated cultures was characterized by flow cytometric analysis. Staining for cells of lymphoid lineage, including NK cells, was absent. The floating cells did however, stain for surface antigens that are known to be associated with mouse DC. These included CD45 (leukocyte common antigen), MHC class I, heat stable antigen (J11D), CD54 (intercellular adhesion molecule-1; ICAM-1), CD11b (MAC-1) and CD44 (nonpolymeric determinant of Pgp.1 glycoprotein). In addition, staining of moderate

intensity was observed for the mouse DC-restricted cell surface markers NLDC-145 (interdigitating cells), 33D1 and CD11c (N418; β2-integrin), for the macrophage antigen F4/80 and for FcγRII (CD32), as described previously (18) for mouse BM-derived DC (data not shown). The GM-CSF stimulated BM-derived cells expressed moderate levels of cell surface MHC class II ($I\text{-}A^b$).

Upregulation of MHC class II, B7-1 and B7-2 expression on GM-CSF + IL-4 stimulated BM-derived DC. Cells stimulated with GM-CSF either alone or in combination with IL-4 were harvested after 8 - 10 days and compared for the expression of surface markers linked with T cell stimulatory function. A marked difference was consistently observed between the two cell populations with respect to the intensity of expression of MHC class II and the CD28 ligands, B7-1 (CD80) and B7-2 (CD86). On cells stimulated with GM-CSF (500 U/ml) alone, MHC class II was positive, B7-1 expression was low, whereas B7-2 was negligible/dim (Fig. 1). An increase in GM-CSF concentration from 500-1000 U/ml augmented the intensity of staining for each of these molecules (data not shown). However, expression of MHC class II, and especially B7-2, was markedly and consistently upregulated on cells treated with both cytokines (Fig. 1). The GM-CSF stimulated MHC class II^+ $B7\text{-}1^{low}$ $B7\text{-}2^-$ and the GM-CSF plus IL-4 induced MHC class II^+ $B7\text{-}1^+$ $B7\text{-}2^+$ populations are subsequently referred to herein as $B7\text{-}2^-$ and $B7\text{-}2^+$ DC, respectively.

MLR stimulatory activity of "immature" ($B7\text{-}2^-$) and "mature" ($B7\text{-}2^+$) DC. The GM-CSF +IL-4 stimulated B10 BM-derived DC that were MHC class $II,^+$ $B7\text{-}1^+$ and $B7\text{-}2^+$ were highly potent inducers of primary allogeneic (C3H) T cell responses in 3-day

primary mixed leukocyte cultures (data not shown). The possibility that this high MLR stimulatory activity was due to "carry over" of IL-4 was excluded, as GM-CSF + IL-4-stimulated B7-2$^+$ DC did not induce syngeneic T cell proliferation. On the basis both of immunophenotype and T-cell stimulatory activity, the B7-2$^+$ cells were considered to be ***mature DC.*** In contrast, GM-CSF stimulated MHC class II$^+$ B7-1^{low} B7-2$^-$ BM-derived cells were much poorer inducers of allogeneic T cell activation and exhibited a comparatively low level of MLR-stimulatory activity. This was consistent with their low level of cell surface B7-2 and was similar to that of freshly-isolated, bulk B10 spleen cells. The B7-2$^-$ cells were therefore considered to be ***functionally immature DC.***

Induction of alloantigen-specific T cell hyporesponsiveness by B7-2$^-$ BM-derived DC. Purified C3H splenic T cells were stimulated initially with either freshly isolated B10 bulk spleen cells or GM-CSF stimulated (B7-2$^-$) or GM-CSF + IL-4 stimulated (B7-2$^+$) B10 BM DC; after resting for 2 days, the primed C3H T cells were restimulated in a secondary MLR with fresh B10, C3H (syngeneic) or BALB/c (third party) splenocytes. As shown in Fig. 2, the T cells stimulated with B7-2$^+$ B10 DC in a primary MLR displayed strong proliferative responses when restimulated with B10 spleen cells. In contrast, T cells exposed initially to B10 B7-2$^-$ BM DC responded only weakly upon restimulation with B10 splenocytes and at a lower level than C3H T cells primed with fresh B10 splenocytes. The same T cells however, displayed proliferative responses to third party (BALB/c) spleen cells, indicating that the hyporesponsiveness was donor-specific (data not shown). The addition of anti-CD28 mAb to primary MLR cultures significantly increased the allostimulatory function of immature BM DC. Furthermore,

priming of allogeneic T cells with B7-2$^-$ DC in the presence of anti-CD28 prevented hyporesponsiveness in secondary MLR.

T cell hyporesponsiveness induced by B7-2$^-$ DC is associated with impaired IL-2 production. To determine whether the induction of hyporesponsiveness in C3H T cells was linked to blockade of IL-2 production, levels of IL-2 were determined by bioassay in supernatants harvested from B10 restimulated T cells primed initially with B10 splenocytes, B7-2$^-$ B10 DC or B7-2$^-$ DC in the presence of anti-CD28. C3H T cells primed with B7-2$^-$ DC failed to produce IL-2 in 2° MLR (data not shown). The presence of anti-CD28 in the 1° MLR however, prevented subsequent unresponsiveness. Hyporesponsive T cells primed by B7-2$^-$ BM-derived DC responded normally to alloantigen + exogenous IL-2 (50 U/ml) added at the outset of the secondary MLR (data not shown). Collectively, these findings strongly suggest the importance of an IL-2 production defect in the induction of the hyporesponsive state.

B7-2$^-$ BM-derived DC prolong cardiac allograft survival. B7-2$^-$ B10 BM-derived DC (2×10^6 i.v.) given 7 days before organ transplant, prolonged the median survival time (MST) of B10 cardiac allografts in C3H mice (Table 1) from 9.5 to 22 days. Third party (BALB/c) B7-2$^-$ BM-derived DC however, also prolonged B10 heart graft survival, although the MST was significantly less than that achieved with donor-specific B7-2$^-$ DC.

DISCUSSION

In contrast to classical mature DC (3), functionally immature, MHC class II^+, B7-1^{dim}, B7-2^- BM-derived DC, propagated in response to GM-CSF induced alloantigen-specific hyporesponsiveness in T cells upon rechallenge in vitro. This hyporesponsive state was prevented by stimulation via the CD28 pathway and reversed by exogenous IL-2. BM DC stimulated with GM-CSF + IL-4 exhibited an overall similar phenotype to the immature DC, but stained intensely for B7-2 in addition to enhanced B7-1 and high MHC class II antigen expression. In contrast to the B7-2^- DC, these cells were very potent allostimulators. Our findings are consistent with those of Hathcock etal (22) who showed that quantitative differences in B7-1 and B7-2 expression on murine APC (B cells) could profoundly affect their contribution to costimulatory function.

Immature MHC class II^+ DC that are B7-2^- may (as is the case with inhibition of B7-2 [22]), allow TCR-mediated signalling events to occur but inhibit the distinct costimulatory signal(s) that are necessary for optimal cytokine production and cytokine-dependent T cell proliferation. Thus, antigen presentation by APC that are unable to deliver adequate costimulatory signals induces neither of the gene transcription factors NF-AT or NF-kB (23).

In principle, the induction of irreversible T cell anergy to donor-specific alloantigens in vivo (tolerance) would eliminate the need for potentially toxic, non-specific immunosuppressive agents which currently constitute first choice anti-rejection therapy. Thus, there has been considerable interest in evidence that blockade of the B7-CD28 T-cell costimulatory pathway is both necessary and sufficient to induce antigen-

specific T cell anergy (24). Although the co-stimulatory signals delivered to T cells by APCs are neither antigen-specific nor MHC-restricted, they determine the outcome of TCR signaling as they mediate cytokine secretion. Co-ligation of the T cell CD28/CTLA-4 receptors (an essential stimulus of T cell activation) (25) by the APC counter-receptors B7/BB1 (CD80) or the recently identified B70/B7-2 (CD86) (22,26,27) results in IL-2 production and T cell proliferation (25,26,27). It is thought that this is achieved either by increased IL-2 mRNA transcription (28,29) or mRNA stability (30). Elicitation of strong IL-2 production by T cells has long been recognized as a characteristic of antigen-presenting DC (31,32).

Although DC express several adhesion and costimulatory molecules (CD11a, CD44, CD54, CD48/58, CD80 and CD86), B7-2 appears to be the major CD28/CTLA-4 ligand and accounts for the costimulation provided by this pathway during in vitro immune responses. Indeed, in a recent study, anti-CD80 (B7-1) mAbs failed to inhibit human dendritic Langerhans cell-induced alloactivation of T cells (33). Early and sustained expression of B7-2 mRNA (within 8 hr) (before maximal B7-1 expression) in activated murine B cells (34), suggests that it may provide a critical signal involved in the decision between activation or inactivation of T cells within 24 hr of stimulation (35,36). Moreover, APCs from mice in which the B7-1 gene has been knocked out express B7-2 and are competent APCs (34).

T cell co-stimulation mediated by CD28 is important in the mediation of transplant rejection and the development of new therapeutic immunosuppressive strategies. Thus the combination of anti-B7 mAb with cyclosporine A induces

alloantigen-specific anergy in vitro (37). CTLA4-Ig, a fusion protein that blocks the CD28-mediated costimulatory signal inhibits T cell proliferation in allogeneic MLR (22,39), and induces hyporesponsiveness to alloantigen in vitro (11). Moreover, it prolongs MHC-mismatched cardiac allograft survival in rats (38,39) and induces donor-specific transplantation tolerance to skin grafts in cardiac-allografted mice (13). Propagation of B7-2$^-$ immature DC with the capacity to induce alloantigen-specific hyporesponsiveness has significant in vivo implications. We have shown that immature DC can be propagated from the liver in response to GM-CSF. Conceivably, the tolerogenicity of liver allografts may be linked, at least in part, to the release of potentially T cell anergizing DC from the liver, a pseudo-haemopoietic organ (40). This is likely to occur under the influence of locally-produced GM-CSF (41) and possibly other growth promoting cytokines. The failure of the B7-2$^-$ DC to induce complete unresponsiveness (anergy) in vitro or indefinite cardiac allograft survival however, suggests that considerations of the cellular therapy of allograft rejection using immature donor-derived DC should include the use of adjunctive immunosuppression, implementing drugs or molecular approaches geared to inhibit costimulatory molecule expression on APC.

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FIGURE LEGENDS

FIGURE 1. FACScan® analysis of MHC class II ($I\text{-}A^b$), B7-1 and B7-2 expression on (above) 8-day GM-CSF and (below) 8-day GM-CSF + IL-4 stimulated B10 BM-derived bone marrow DC. Further details are provided in the Methods or have been described (17). "Shaded" profiles denote appropriate Ig isotype subclass controls. The result is representative of 3 separate experiments.

FIGURE 2. Stimulation with B7-2$^-$ BM-derived DC during a primary MLR induces hyporesponsiveness upon restimulation in secondary MLR. C3H splenic T cells (2 x 10^6/ml at 1:2 [S:R]) ratio were stimulated during a primary MLR with either γ-irradiated (O) GM-CSF stimulated B10 BM-derived DC (B7-2$^-$), (●) GM-CSF + IL-4 stimulated B10 BM-derived DC (B7-2$^+$) or (▵) fresh B10 spleen cells. The T cells were rested for 2 days then restimulated with graded concentrations of freshly-isolated B10 spleen cells. The results are expressed as mean counts per minute (cpm) $\pm$ 1SD and are representative of four separate experiments.

Table 1 Influence of donor-specific GM-CSF stimulated bone-marrow (BM)-derived DC (B7-2$^+$) on cardiac allograft survival

Cells injected (day-7)	Graft survival times (days)	MST (days)	
None	8,12,13,13,8,9,10,8	9.5	
B10 BM	12,12,12,12	12	
B10 BM-DC	19,19,19,22,22,23,27,35	22	$p < 0.02$
C3H BM-DC (syngeneic)	12,12,13,13	12.5	
BALB.c BM-DC (third party)	17,17,12,16,20,19	17	
B10 spleen-DC (B7-2$^+$)	8,8,9	8.5	

EXPRESSION OF MHC II (I-A^b), B7-1 AND B7-2 ON GM-CSF AND GM-CSF + IL-4 STIMULATED BM - DERIVED DC

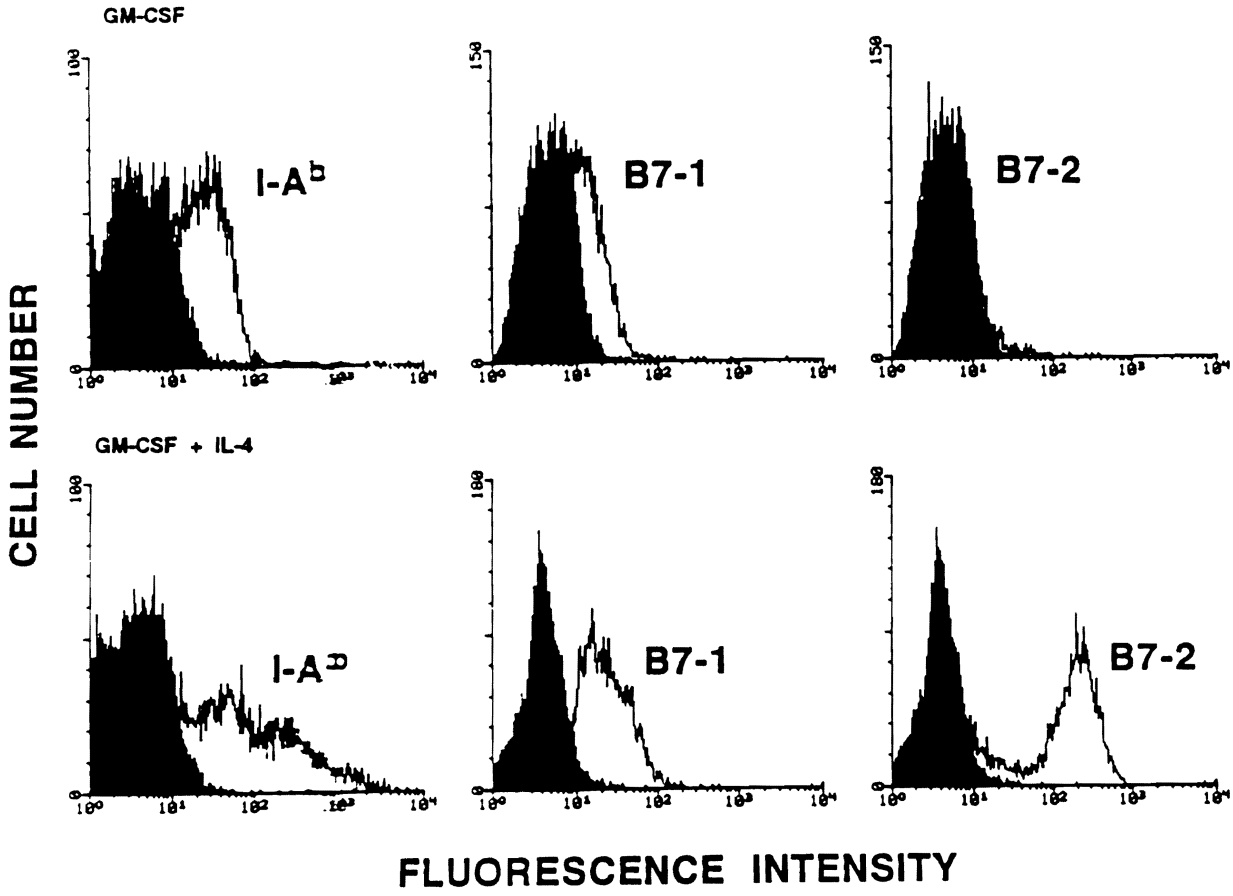

Figure 1

Proliferation of C3H T cells Primed with B7-2⁻ or B7-2+ B10 BM DC and Restimulated with B10 Spleen Cells

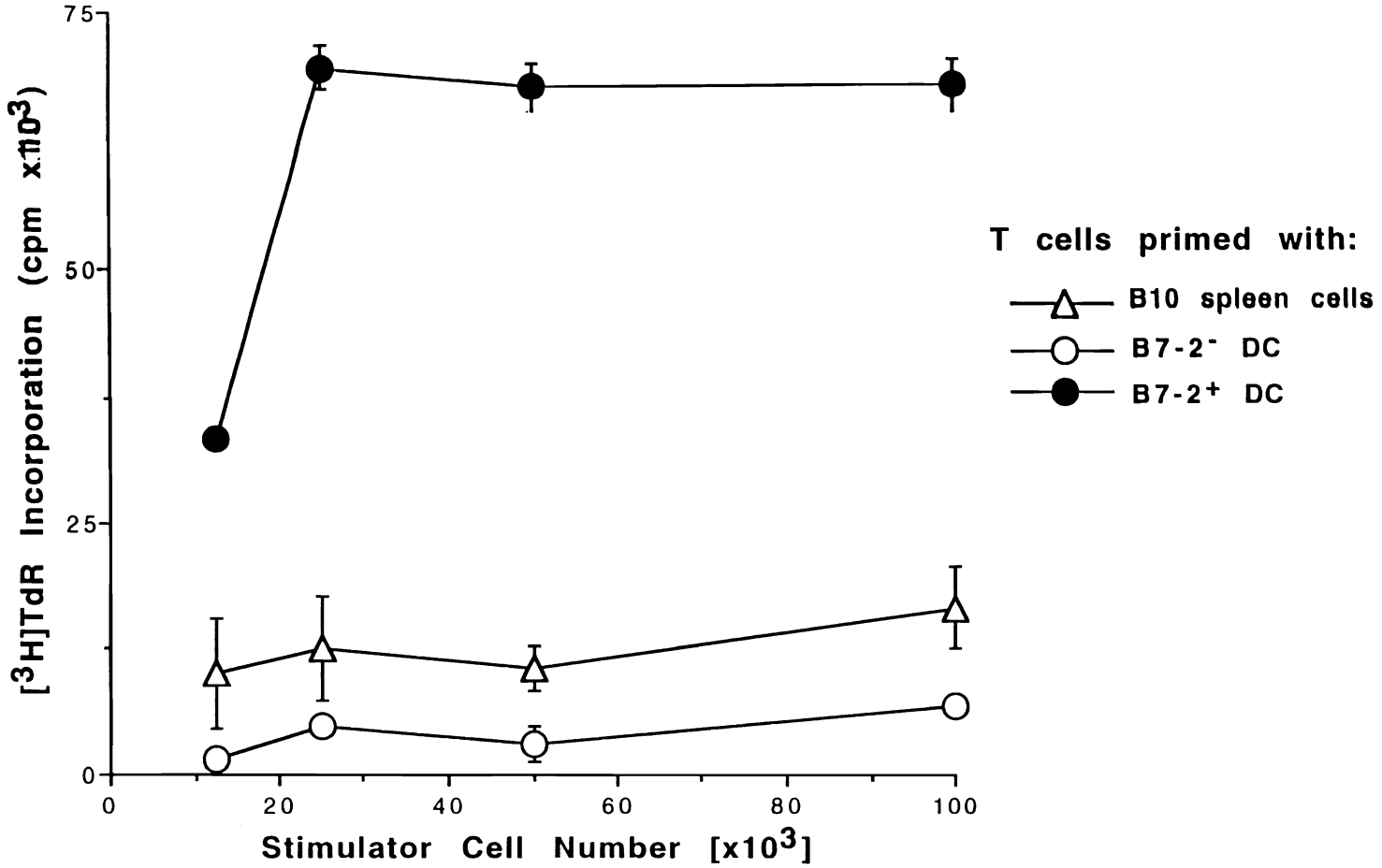

Figure 2