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The Effects of Interleukin-1, Granulocyte Macrophage Colony-Stimulating Factor, and Tumor Necrosis Factor-α on Cultured Human Langerhans Cells and Cortical Thymocytes

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Abstract

Langerhans cells (LC) are bone marrow derived residents of the epidermis which express the CD1a surface antigen and undergo a variety of phenotypic and functional changes *in vitro*. To determine the effects of the epidermally associated cytokines granulocyte macrophage colony-stimulating factor (GM-CSF), Tumor Necrosis Factor-α (TNF-α), and Interleukin-1 (IL-1) on LC phenotype *in vitro*, epidermal cells were cultured in the presence of these cytokines and the percentage of cells expressing CD1a was determined by flow cytometry. By the fifth day, the percentage of cells expressing CD1a in TNF-α and control cultures was about half of the starting value, while in IL-1 and GM-CSF cultures CD1a expression was respectively higher and lower than control. To determine if these effects on CD1a expression are generalizable, expression of CD1a by human cortical thymocytes was also studied. GM-CSF decreased and IL-1 increased thymocyte CD1a expression. In additon, these two cytokines had opposite effects on class I major histocompatibility complex (MHC) protein expression, suggesting that these two antigens are reciprocally regulated. To correlate these phenotypic changes with function, LC precultured with cytokines were tested for

their ability to stimulate allogeneic T-cell proliferation. GM-CSF treated cultures caused 2 to 3 times more T-cell proliferation than control cultures. Addition of antibodies reactive with class I and class II MHC molecules blocked T-cell proliferation while antibodies to CD1a did not. We conclude that GM-CSF, a cytokine that is produced by keratinocytes and thymic nonlymphoid cells *in vitro*, can decrease CD1a expression by thymocytes and LC, while increasing class I MHC expression by thymocytes. Furthermore, GM-CSF enhances the ability of human LC to stimulate allogeneic reactions *in vitro*. This ability can be blocked by antibodies to both class I and class II MHC molecules.

Introduction

Langerhans cells (LC) are a minor population of epidermal cells that have been the subject of a large body of research since their discovery by Paul Langerhans in 1868. Langerhans believed that the dendritic cells he had discovered with gold chloride staining were intraepithelial receptors for extracutaneous signals to the nervous system [1], and such a theory prevailed for a century. In 1968, however, Breathnach et al proved that murine Langerhans cells do not originate in the neural crest, by demonstrating that murine skin deprived of its neural crest components still contained LC [2]. Further experiments carried out by Frelinger et al using murine radiation chimeras

reconstituted with allogeneic bone marrow demonstrated that LC originate in bone marrow [3]. These findings were confirmed in similar experiments by Katz et al in the same year [4]. Subsequent studies in humans after male to female bone marrow transplantation, have established the bone marrow origin of human LC by demonstrating the presence of exclusively Y chromosome-positive LC (donor LC) in the skin biopsies of female bone marrow transplant recipients [5,6].

During the 1970's a large body of evidence accumulated that phenotypically linked LC to cells of the macrophage/monocyte lineage. Both types of cells express Fc and C3 receptors [7], HLA-DR (Class II major histocompatibility complex) molecules [8,9], membrane ATPase activity [11], and have the ability to migrate [10]. Based on these findings, as well as ultrastructural evidence [12,13], Katz et al has suggested that monocytes are the most likely precursors of epidermal LC [4], however formal proof of a specific LC precursor is lacking.

In contrast to the similarities between LC and macrophage/monocytes, several phenotypic and structural features distinguish LC from all other cells studied to date. Most striking of these features is a cytoplasmic structure known as the Birbeck granule. In 1961, Birbeck published the first electron micrographs of epidermal LC in which a unique membrane bound granule was observed. Birbeck described this cytoplasmic granule as a linear structure with rounded ends and a striated line running down the center [14]. This is the typical "tennis

racket" shape that is usually associated with Birbeck granules. Recent evidence has suggested that these granules are involved in receptor mediated endocytosis along with lysosomes, coated pits, and coated vesicles [15,16,36], and they remain the gold standard for the identification of epidermal LC.

Epidermal LC are also unique in their surface antigen profile. As mentioned above, they express class II MHC protiens, however, there is controversy as to whether they express very low levels of class I MHC molecules [17,71], or are totally deficient in class I MHC protein expression [18,19]. This relative lack of class I MHC protein expression by LC is quite unusual and is shared only with certain subsets of cortical thymocytes [37]. In addition, LC express the CD1a surface antigen which is a glycoprotein with a relative mass of 49,000 [20,21]. CD1a has also been called "T6" because it is recognized by the OKT6 monoclonal antibody [21], and is biochemically identical to Human Thymocyte Antigen 1 (HTA-1) [22,23] which is expressed by cortical thymocytes [24,25]. In the further biochemical characterization of the CD1a molecule several similarities to class I MHC protiens have been noted. CD1a and class I MHC molecules share serologically defined epitopes [26] and have biochemically similar structures including three extracellular domains named α-1, α-2, and α-3 [26-35]. CD1a and class I MHC protiens both associate with β-2 microglobulin through their α-3 domain [29-31], and both have been reported to associate with the CD8 thymocyte surface molecule [32,33]. In addition, the predicted secondary structure

of the α-1 and α-2 domains of CD1a is very similar to the antigen binding pocket of HLA-A2, which is a well characterized class I MHC molecule [27,28]. As a result of these similarities, it has been argued that CD1a represents a novel type of class I MHC molecule [30,31], and as such may be involved in the presentation of antigen to T-cells [26-33]. While presentation of antigen by CD1a has not been proven, CD1a does appear to be part of a receptor site since it is internalized via coated pits, coated vesicles, and endosomes in ways characteristic of receptor mediated endocytosis [36]. It is also intriguing that LC and cortical thymocytes are the only cells expressing CD1a and are the only nucleated somatic cells deficient in class I MHC protein expression [37,38]. The structural similarities, together with the reciprocal anatomic distribution of CD1a and class I MHC molecules, suggests that these molecules may perform either similar, or mutually exclusive functions.

While recent work has focused on the precise role of immune function associated molecules such as CD1a expressed by LC, the function of LC themselves has been studied and debated since their discovery. As mentioned above, Langerhans himself initially believed that these cells were part of the peripheral nervous system [1], however, in 1882 he published a short correction of his original paper of 1868 stating "I am now convinced . . . that my cells are in no way essential for nerve endings" [39]. He appearently based this correction on the histological work of Mojsisovics and Merkel [39]. Langerhans' retraction went largely

unnoticed, and little progress was made toward the understanding of LC function for more than a century. In 1973, however, Silberberg described the apposition of LC and mononuclear lymphocyte-like cells in man, within 4-6 hours of the topical application of mercury bichloride, which is known to induce contact hypersensitivity [10]. Furthermore, this apposition was identified only in subjects with allergic reactions to mercury bichloride, and not in subjects with primary irritant reactions, or no reaction to mercury bichloride [10,40,41]. In addition, it has been shown that contact sensitivity to a specific allergen can be passively transferred by lymph node cell suspensions from sensitive to naive animals, and after dermal challenge with the allergen, allergen bearing LC are found in lymph nodes of the formerly naive animals [42,46]. Subsequent studies have proven that LC can pick up antigen in the skin and carry it by way of dermal lymphatics, to draining lymph nodes [42-45]. These findings firmly established an immunological role for LC and strongly suggested that they may be involved in the presentation of antigen to T-cells in lymph nodes. *In vivo* proof of the antigen presenting capacity of LC has accumulated from two other types of observations as well. In the first, it has been noted that cutaneous surfaces devoid of LC do not support the development of contact hypersensitivity after application of allergen [59-61]. In the second, LC deficient allografts such as tape-stripped skin and cornea, have been shown to lack the ability to induce alloimmune reactions [62-65]. More recently Cruz et al have

reported that intravenously infused murine LC migrate preferentially to skin where they can participate in the induction of contact hypersensitivity [66]. In summary it is known that LC originate in bone marrow, migrate to the epidermis where they pick up antigen, and then transport antigen to draining lymph nodes where they stimulate antigen specific immune responses.

The *in vivo* findings discussed above have been supported by many studies of LC *in vitro*. While epidermal LC do not survive in culture for more than 6 to 9 days [69,70], recent interest has focused on marked changes in their observed phenotype and function *in vitro*. As mentioned above, freshly isolated LC express class II MHC [8,9] and CD1a surface antigens [21] and little or no class I MHC protein [17-19]. However, when maintained in culture for 2 to 3 days, LC show increased expression of class I MHC and class II MHC antigens, while losing CD1a positivity [71,72]. These phenotypic changes have been related to changing functional activities of LC such as processing antigen in the skin, and then presenting it in draining lymph nodes [75]. Studies of human LC have shown that they can substitute for macrophages in presenting a variety of antigens such as tuberculin protein, herpes simplex virus proteins, trichophytin, and nickel sulfate to autologous T-cells from sensitized donors [47-50]. In addition, studies in both animals and humans have shown that LC enriched epidermal cell suspensions, but not LC depleted suspensions, induce a strong proliferative response from resting allogeneic T-cells in mixed epidermal-lymphocyte

reactions (MELR's) [50,51]. This stimulation has been shown to occur in an MHC class II restricted fashion [52-53]. Since it is the CD4 glycoprotien which recognizes antigen complexed to MHC class II molecules [54-58], this stimulation probably involves CD4+ cells, such as T-helper lymphocytes. Functional studies comparing the abilities of freshly isolated and cultured murine LC have shown that freshly isolated LC actively present protein antigens such as myoglobin to specifically primed T-cells, but are relatively poor stimulators of allogeneic T-cells in MELR's [73]. However, after culture for two to three days, murine LC present protein antigen only very weakly, while their ability to stimulate allogeneic T-cells increases roughly ten fold [73-75]. Similar studies by Teunissen and Romani have shown that the ability of human LC to stimulate allogeneic reactions increases two to ten fold after 3-5 days of culture [76,71]. Teunissen has suggested that this increased ability to stimulate allogeneic T-cells may be due to increased MHC class II protein expression on cultured LC [76], however, the relationship between the phenotypic and functional changes seen in cultured LC has not been clearly defined. All of the above observations from *in vitro* and *in vivo* studies have led to the conclusion that LC are the primary antigen presenting cells of the skin [67], and have implicated LC as the most peripheral component of a unique collection of cells and lymphoid organs called skin-associated lymphoid tissues (SALT) that function as a group to perform the specialized immune requirements of the skin [68].

Most recently, reports from work in murine systems have shown that epidermally associated cytokines such as Interleukin-1 (IL-1), Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), and Tumor Necrosis Factor-α (TNF-α), may influence LC phenotype and function *in vitro* [69,77-79]. Specifically, Witmer-Pack and collegues have shown that the addition of GM-CSF to murine LC cultures has three effects over a 2-3 day culture period: cell viability is increased, LC become larger and more dendritic, and their stimulatory ability for allogeneic T-cells increases 10 to 20 fold [77]. Similar work by Heufler et al using several measures of stimulatory ability has comfirmed that it is the direct effect of GM-CSF, and not simply improved viability which leads to this enhancement in function [69]. In addition, they showed that a combination of IL-1 and GM-CSF added to murine LC cultures leads to a 2 fold further increase in stimulatory capacity compared to LC cultured with GM-CSF alone [69]. TNF-α has been shown to enhance murine LC viability *in vitro*, however, TNF-α treated LC remain relatively poor stimulators of allogeneic T-cells [78]. In phenotypic studies Belsito et al have recently reported an enhancement of MHC class II antigen expression by murine LC cultured in the presence of either IL-1, GM-CSF or TNF-α [79]. Evidence of the effects of these cytokines on human epidermal cells is scarce. However, Walsh et al report that IL-1 can induce CD1a expression on human gingival epithelial cells [80]. All of these *in vitro* findings may have important implications for the regulation of LC phenotype

and function *in vivo* since it is known that cultured human keratinocytes produce IL-1 [79], and GM-CSF [82]. It has also been shown recently that TNF-α message is detectable in human epidermal mRNA preparations [Longley et al in preparation]. When injected intraveneously in mice, TNF-α targets specifically to the skin [83], indicating the presence of receptors for TNF-α, and suggesting that this cytokine plays a role in the biology of the skin. To determine how these cytokines affect the phenotypic characteristics of human LC *in vitro*, we cultured purified LC from normal human skin with IL-1, GM-CSF, and TNF-α, and compared CD1a expression with untreated cultures. In a similar set of experiments we cultured human cortical thymocytes, which also express CD1a, with the same cytokines and measured both CD1a and class I MHC expression. To assess the functional effects of these cytokines, we compared the ability of treated and untreated human LC to stimulate proliferation of allogeneic T-cells, and attempted to block this proliferation with a variety of monoclonal antibodies. T-cell stimulation was measured through standard techniques of the MELR.

Materials and Methods

Isolation of Langerhans Cells

Normal human skin was obtained from elective plastic surgery procedures and trimmed to the level of the papillary dermis. Skin

was then incubated in Tyrodes solution with 10 mg/ml Dispase (Boerhinger Mannheim, W. Germany) overnight at 4°C and then at 37°C for 20 minutes. Dispase is a neutral protease which selectively digests fibronectin and type IV collagen [87] thus allowing sheets of epidermis to be easily separated from the dermis and assuring that only epidermal cells were studied. Epidermal sheets were then incubated at 37°C for 15 minutes in 0.125% Trypsin (Gibco Labs Inc., Grand Island, New York), vortexed at low speed for 1-2 minutes and filtered through three layers of sterile gauze. The resulting epidermal cell suspension was then layered over Histopaque-1077 (Sigma Diagnostics, St. Louis, Mo.) at 4°C and centrifuged at 300 g's for 20 minutes. Cells remaining at the interface were collected, viability assessed by trypan blue exclusion, and the percentage of cells expressing CD1a measured by flow cytometry after staining with OKT6 monoclonal antibody (from ATCC hybridoma # CRL 8020), and FITC conjugated goat anti-mouse secondary antibody (Becton Dickinson, Mountain View, CA). This technique reliably yielded 97-100% living cells and a maximum of 30% CD1a positivity. In some LC experiments, a further enrichment for CD1a positive cells was carried out by staining interface cells with an anti-class I MHC monoclonal antibody (anti-HLA-A,B,C from the W6/32 hybridoma ATCC # HB95) and counterstaining with an iron conjugated goat anti-mouse secondary antibody (Advanced Magnetics Inc., Cambridge, MA.). These cells were then precipitated to a magnetic plate (Advanced Magnetics Inc.) and nonmagnetic cells were washed

from the plate. This procedure increased the percentage of CD1a positive cells from a maximum of 30% with Histopaque enrichment alone, to a maximun of 50%.

Langerhans Cell Cultures

Cells were then plated out at $1X10^6$ cells/ml in 24 well flat bottomed plates (Corning Glass Works, Corning, NY) in RPMI 1640 (Gibco Labs Inc.), 10% defined fetal bovine serum (HyClone Labs Inc., Logan, UT), 1% Penicillin G/Streptomycin/Amphotericin B (Gibco Labs Inc.) and 10ng/ml of recombinant human IL-1, GM-CSF (gift of Dr. Steven Gillis, Immunex Corp., Seattle, WA.), or TNF-α (courtesy of Dr. Thomas Kupper, Yale School of Medicine, Dept of Dermatology) versus control. Viability and CD1a positivity were assessed on day 0, 2, 3, and 5 of culture by flow cytometry.

Thymocyte Isolation

Thymus glands were obtained from newborn infants during the course of corrective cardiac surgery, minced, suspended in RPMI 1640 (Gibco Labs Inc.), and filtered through sterile gauze. The resulting cells were purified on a Histopaque-1077 (Sigma Diagnostics) gradient as for LC, and then cultured at $1X10^7$ cells/ml with conditions and cytokines as for LC. In a series of depletion experiments, CD1a, or class I MHC positive cells were depleted from thymus gland cell suspensions before culture using the appropriate monoclonal antibody, and the magnetic technique detailed above. In all cultures, the percentage of cells expressing

CD1a and class I MHC was determined on day 0 and 8 by flow cytometry.

Preparation of T-cells

Resting allogeneic T-cells for mixed cultures were prepared as follows: Peripheral mononuclear cells (PMN) were obtained from the blood of healthy volunteers through Histopaque density gradient centrifugation as above. PMN were then suspended in RPMI 1640 (Gibco Labs Inc.), 10% defined fetal bovine serum (HyClone Labs Inc.), 1% Penicillin G/Streptomycin/Amphotericin B (Gibco Labs Inc.) at $1X10^7$ cells/ml and monocytes allowed to adhere to a plastic culture flask (Corning Glass Works) for 3-12 hr.s. Medium containing nonadherent cells (lymphocytes) was then decanted and the cells stained with an anti-class II MHC protein antibody which reacts with HLA-DR, DP, (and possibly DQ) framework determinant produced by the IVA12 hybridoma (ATCC #HB 145). Resting T-cells were then negatively selected with the magnetic procedure described above. This procedure reliably yielded a cell suspension of 94-97% T-cells as assessed by expression of T-cell receptor associated antigen (CD3) by flow cytometry.

Mixed Epidermal Cell-Lymphocyte Cultures

Epidermal cells were prepared and cultured as above except that they were plated out in triplicate over a range of 0-3000 LC, in 200 microliters per well, in 96 well round bottomed plates

(Corning Glass Works). The culture medium was the same as for LC cultures, with either GM-CSF, IL-1, or TNF-α at 10 ng/ml. On day 3 of culture (72 hr.s), plates were centrifuged for 5 minutes at 300 g and the culture medium replaced with medium containing 10^5 purified allogeneic T-cells without cytokines. These mixed epidermal cell-lymphocyte cultures were then incubated for 4 more days, pulsed with 1 microCurie of ^{3}H-Thymidine (Amersham Inc., UK) per well, harvested 16 hr.s later and counts per minute (CPM's) assessed with an LBK scintillation counter.

Blocking Studies

MELR's were performed as above, however monoclonal antibodies to either class I MHC (anti-HLA-A,B,C, produced by the hybridoma ATCC # HB95), class II MHC (ATCC # HB145), or CD1a (ATCC # CRL8020) were added to each set of cultures at the time of T-cell addition. T-cell stimulation was measured by ^{3}H-thymidine incorporation as above.

Statistical Analysis of Results

For LC cultures, experimental and control mean values with standard errors were calculated and compared using the Student's t-test. For thymocyte cultures all experimental values were expressed as percents of control with control values set to 100%. Averages and standard errors of experimental values were then calculated and compared using a one way analysis of variance

(ANOVA). In MELR's experimental and control means and standard errors were calculated and compared using an ANOVA as above.

Results

Langerhans Cell Cultures

Viability of epidermal cells decreased in a predictable manner such that nearly all cells were dead by trypan blue exclusion by day 6 (Figure 1). After 2 days of culture, CD1a expression among viable cells in medium alone, GM-CSF, IL-1, and TNF-α had dropped from 30% upon isolation to 12.4%, 13.5%, 13.0%, and 11.4% respectively. By day 5 of culture, CD1a expression among control cells had dropped to 9.75±1.2%, while TNF-α and IL-1 cultured cells were 11.4±1.2% and 14.5±0.4% positive respectively. However, only 4.2±0.3% of GM-CSF cultured cells were CD1a positive by day 5 (Figure 2). IL-1 significantly prolonged the expression of epidermally associated CD1a antigen compared to untreated cultures ($p=0.02$), while GM-CSF significantly decreased CD1a expression ($p=0.01$). TNF-α did not significantly alter CD1a expression in any cultures.

Thymocyte Cultures

The percentage of cells expressing CD1a or class I MHC antigens after 8 days was determined after culture with the indicated cytokines. The number of cells expressing either

antigen in cytokine treated cultures was divided by the number of cells expressing the same antigen in untreated cultures and the resulting value multiplied by 100 to give the percent of control cells expressing CD1a or class I MHC antigen. Figure 3 shows the mean values obtained from 5 such experiments. The percentage of cortical thymocytes expressing CD1a on day 8 was 124±12.7% (TNF-α), 157±33% (IL-1), and 78±7.4% (GM-CSF) of control, while the percentage of cells expressing class I MHC was 109±12%, 92±8.0%, and 148±34% of the control cultures respectively. Thus GM-CSF treatment was associated with accelerated loss of CD1a expression from thymocyte cultures ($p=0.03$), and increased class I MHC expression ($p=0.02$), compared to control. IL-1 was associated with effects opposite to those of GM-CSF, maintaining the percentage of cells expressing CD1a above control ($p=0.01$) and decreasing the percentage of cells expressing class I MHC, however this effect was not significant ($p=0.6$). TNF-α showed no significant effect on either CD1a or class I MHC expression, however the small changes it did cause were similar in trend to those of IL-1.

In a well known model of surface antigen expression during thymocyte maturation, very early thymocytes are thought to be CD1a negative/class I MHC positive, but during differentation become CD1a positive/class I MHC negative. These cells again become CD1a negative/class I MHC positive as they mature fully and exit the thymus [37]. There is also a small, well documented population of CD1a negative/class I MHC negative (double

negative) thymocytes which may represent cells in transition between the major populations listed above [37]. In order to more clearly define the effects of cytokines on surface antigens at various points along this linear differentiation pathway, we performed the same experiments with thymocyte populations that had been depleted of CD1a and class I MHC positive cells as detailed above.

Figure 4 shows the result of experiments in which CD1a positive cells were magnetically depleted from thymocyte suspensions prior to culture. In these experiments CD1a expression was 108±3.8% (TNF-α), 146±16% (IL-1), and 64±6% (GM-CSF) of control, while class I MHC expression was 139±12%, 220±17%, and 96±8.8%, respectively. Therefore, GM-CSF prevented CD1a expression by CD1a negative/class I MHC positive cells ($p=0.01$). However, GM-CSF had no effect on class I MHC expression in this population of thymocytes ($p=0.8$). This finding implies that GM-CSF may either block the maturation of CD1a negative/class I MHC positive cells to the CD1a positive/class I MHC negative phenotype, or accelerate development of the most mature CD1a negative/class I MHC positive thymocytes. In CD1a depleted cultures, IL-1 increased the percentage of cells expressing CD1a compared to control ($p=0.03$), an effect opposite to that of GM-CSF, and unlike undepleted cultures, IL-1 significantly increased the expression of class I MHC ($p=0.08$). These effects could be explained if IL-1 was a general accelerator of thymocyte maturation moving cells both into and

out of the CD1a positive/class I MHC negative state. Another explanation could be that IL-1 increases the antigen expression and/or viability of thymocytes without effecting maturation. In this case, the increased percentage of cells expressing these two antigens could be explained by larger numbers of viable cells expressing antigen, or more cells expressing detectable levels of antigen. TNF-α again produced insignificant changes in antigen expression in these cultures.

In a final set of thymocyte experiments, class I MHC positive cells were depleted prior to culture yielding thymocyte populations that were 80-90% CD1a positive, and containing small numbers of CD1a negative/class I MHC negative cells. The results of these experiments are shown in figure 5. CD1a expression among thymocytes was 126±8.4% (TNF-α), 152±17% (IL-1), and 94±3.9% (GM-CSF) of control, while class I MHC expression was 122±8.6%, 108±4, and 180±4.0% of control, respectively. In these experiments IL-1 significantly increased the percentage of cells expressing CD1a ($p=0.03$), and GM-CSF significantly increased class I MHC expression ($p=0.08$), while no other results were significant. These findings lend additional support to the hypothesis that GM-CSF accelerates the maturation of CD1a positive/class I MHC negative cells to the CD1a negative/class I MHC positive phenotype. In addition these results suggest that IL-1 may influence the differentation of CD1a negative/class I MHC negative cells to the CD1a positive/class I MHC negative phenotype.

To summarize the results of these three sets of experiments, GM-CSF tended to decrease CD1a expression compared to control in undepleted, CD1a depleted, and class I MHC depleted cultures, however the effect in this latter set of cultures was small and not statistically significant ($p=0.6$). IL-1 increased CD1a in all three sets of cultures, while TNF-α had no effect on CD1a expression. GM-CSF increased class I MHC expression compared to controls in undepleted and class I MHC depleted cultures, but not in CD1a depleted cultures. This is exactly the opposite effect and pattern of significance compared to GM-CSF's effect on CD1a. IL-1 increased class I MHC expression only in CD1a depleted cultures, and as in studies of CD1a, TNF-α had no effect on class I MHC expression. This complex pattern of results is summarized in table 1.

In both thymocyte and LC cultures, GM-CSF tended to decrease CD1a expression compared to control, while IL-1 increased CD1a expression. TNF-α had no effect on CD1a expression in either thymocyte or LC cultures. In undepleted thymocyte cultures GM-CSF tended to reciprocally effect CD1a and class I MHC, decreasing CD1a expresion while enhancing class I MHC expression.

Functional Studies of Langerhans Cells

As seen in figure 6, LC precultured for 72 hours with GM-CSF were more potent stimulators of allogeneic T-cell proliferation than cells cultured with IL-1, TNF-α or no cytokine. Specifically,

GM-CSF treated LC produced significantly more allogeneic T-cell proliferation than LC cultured under all other conditions. This was true at all doses of LC tested ($p \leq 0.02$), except at 300 LC where proliferation was the same as control ($p=0.5$). LC cultured with IL-1 showed no significant increase in proliferation at any dose tested, while TNF-α treated LC produced less proliferation than control, but only at a dose of 900 LC ($p=0.05$). Figure 7 shows the results of subsequent experiments concentrating on the effect of GM-CSF, and extending the doses of LC used to a maximum of 3,000. In these cultures GM-CSF treated LC were more potent stimulators of allogeneic T-cells than control cells at $p \leq 0.01$ for all doses of LC tested except at 250 LC where $p=0.05$. Also of note is that there was no significant difference in the amount of T-cell proliferation seen in GM-CSF and control cultures in which there were no LC even though these wells were treated as if they did contain LC during the 72 hour pretreatment period. This observation precludes the possibility that it is the presence of contaminating GM-CSF that is solely responsible for the enhanced T-cell stimulation seen in GM-CSF treated cultures. In addition, there was a direct relationship between the number of LC in culture and the level of T-cell proliferation, regardless of the culture conditions, indicating that GM-CSF augments LC ability to stimulate allogeneic reactions on a cell by cell basis.

Blocking Studies

Figure 8 shows the results of MELR's in which all Langerhans cells were pretreated for 72 hours with GM-CSF. At the time of T-cell addition, however, antibodies reactive to either CD1a, class I MHC, or class II MHC were added to culture. In these cultures the addition of anti-CD1a had no effect on allogeneic T-cell stimulation. In cultures treated with anti-class II MHC, however, T-cell stimulation was inhibited in cultures containing 1500 LC or less ($p \leq 0.02$), while at higher doses of LC, addition of anti-class II MHC antibody did not alter levels of T-cell stimulation. Most interestingly, when anti-class I MHC antibody was added to culture there was an inhibition in T-cell stimulation at all doses of LC tested ($p \leq 0.02$).

Discussion

The study of LC *in vitro* has long been hampered by difficulties in purifying large numbers of intact cells from the epidermis. By modifying the methods used by a variety of different investigators, we have developed a technique for rapidly isolating human LC without altering their surface phenotype. Many methods for LC isolation involve long incubations of skin with the enzyme trypsin to obtain epidermal cell suspensions [84], followed by LC enrichment by a variety of techniques. These include "panning" [77], density gradient centrifugation [76], and fluorescence activated cell sorting [85]. While these techniques yield very

pure populations of LC, they are often long, expensive, and plagued by the uncertain effects of trypsin on LC surface proteins.

Most recently Hanau et al have described an immunomagnetic selection technique where by epidermal cell suspensions are stained with a CD1a reactive antibody, and then counterstained with a secondary antibody that is conjugated to small iron beads. Cell suspensions are then precipitated to a magnetic plate and CD1a negative cells washed from the plate. This procedure results in a very pure population of LC that can be obtained quickly and economically [86]. However, unless the amount of magnetic material used is carefully titrated, the resulting LC are rendered useless for *in vitro* functional studies due to the iron beads bound to their surface. By incubating skin with the enzyme Dispase which is known to digest only fibronectin and type IV collagen [87], we have obtained relatively unmodified epidermal cell suspensions. These suspensions are then enriched for LC by a combination of density gradient centrifugation, and a negative immunomagnetic selection that depletes keratinocytes without altering LC. This technique is detailed above and routinely yields epidermal cell suspensions 30-50% CD1a positive in 4 to 5 hours.

Our phenotypic studies of both LC and cortical thymocytes suggest that expression of CD1a antigen can be altered by IL-1, and GM-CSF. In particular, GM-CSF tended to accelerate the loss of CD1a from LC and thymocyte cultures, while IL-1 maintained it. The effects of these two cytokines on class I MHC expression in thymocyte cultures seemed to be the opposite of their effects

on CD1a. That is to say, GM-CSF maintained class I MHC expression above control, while IL-1 accelerated its loss from nondepleted thymocyte cultures. These results lend credence to the notion that CD1a and class I MHC are reciprocally regulated and are consistant with the observation that CD1a and class I MHC are reciprocally expressed *in vivo* [38,39]. Moreover, because these cytokines can be produced by keratinocytes [79,82] these observations suggest that GM-CSF and IL-1 may play similar roles *in vivo*. While we have shown that the loss of CD1a antigen by human LC can be modulated by IL-1 and GM-CSF, for technical reasons we were unable to study the effects of these cytokines on LC MHC class I expression. As mentioned above however, a recent study by Romani et al, has shown that the loss of CD1a expression by human LC is accompanied by a rapid increase in both class I and class II MHC expression [71]. There remains however, no published observations of the effects of epidermally associated cytokines on class I MHC expression by LC *in vitro*.

Our phenotypic results are consistant with previous work in murine systems showing that loss of CD1a analogs can be accelerated *in vitro* by exogenous GM-CSF [69,77]. These studies also report that GM-CSF enhances the viability of murine LC *in vitro*. In our study there was no significant difference in viability among cells cultured with IL-1, GM-CSF, TNF-α, or control as assessed by trypan blue exclusion. This may represent an important difference between the behavior of human and murine LC *in vitro*. We are aware of only one study of the effects

of cytokines on human LC; Walsh et al have shown that culturing CD1a depleted human gingivial cells with IL-1 induces expression of CD1a in these cultures [80]. Our data support this finding in both human LC and human thymocyte cultures and, as discussed below, we have shown that CD1a depleted thymocytes can be induced to express CD1a after treatment with IL-1, much as gingivial cells did in Walsh's study.

In our initial experiments on the effects of GM-CSF, IL-1, and TNF-α on CD1a and class I MHC expression by unfractionated thymocytes, we found that GM-CSF was associated with decreased expression of CD1a and increased expression of class I MHC, while IL-1 was associated with increased expression of CD1a. The significance of these effects during thymocyte differentiation are unclear, however, due to the complexity of the thymic microenvironment. It is known, however, that thymocyte differentiation takes place in a linear fashion with respect to surface antigen expression. In particular, it is believed that bone marrow derived prothymocytes enter the thymus where they are initially CD1a negative/class I MHC positive. During differentiation, they become CD1a positive/class I MHC negative, and finally as they develop into mature T-cell subsets, they once again become CD1a negative/class I MHC positive [37]. In order to isolate subsets of thymocytes at various points along this pathway and more precisely determine the effects of cytokines during thymocyte development, we performed the CD1a and class I MHC depletion experiments detailed above. While the results of

these depletion experiments must be interpreted with caution, the pattern of effects seen indicates that GM-CSF may prevent CD1a expression by cells of the CD1a negative/class I MHC positive phenotype, and enhance expression of class I MHC by cells of the CD1a positive/class I MHC negative phenotype. In general, this pattern suggests that GM-CSF may be involved in the reciprocal regulation of CD1a and class I MHC molecules. When explained in the context of the thymocyte differentiation model mentioned above, these results imply that GM-CSF may either block the development of the CD1a positive/class I MHC negative phenotype, and/or accelerate the development of the CD1a negative/class I MHC positive phenotype.

The effects of IL-1 on CD1a and class I MHC expression in thymocyte cultures are in general opposite to those of GM-CSF, in that IL-1 increases CD1a expression by cells expressing class I MHC (CD1a positive/class I MHC negative and CD1a negative/class I MHC positive thymocytes), however IL-1 also increases CD1a expression by CD1a positive/class I MHC negative thymocytes. IL-1 has no effect on class I MHC expression unless CD1a positive cells are depleted, in which case IL-1 increases class I MHC expression. The observed effects of IL-1 on thymocyte surface antigen expression are hard to explain within the context of the thymocyte differentiation model discussed above, because IL-1 tended to increase both CD1a and class I MHC expression by CD1a negative/class I MHC positive thymocytes. This effect could be accounted for if IL-1 had a significant effect on CD1a

negative/class I MHC negative thymocytes in transition between the major populations, or if IL-1 enhances the antigen expression and/or viability of thymocytes *in vitro*, in which case more cells may appear to be expressing both antigens compared to control. We are currently performing experiments using magnetically selected CD1a negative/class I MHC negative thymocytes to explore the effects of IL-1, as well as GM-CSF and TNF-α on this population of cells.

Also of note is that TNF-α produced only weak changes in CD1a and class I MHC expression which were not statistically significant. This is not suprising because while TNF-α production has been shown in human thymocytes [92], its effects may have been obscured since TNF-α can induce GM-CSF production in a variety of cell types [91]. Thymocyte differentiation is influenced by a variety of factors including cell-cell contact [93] and cytokines released by epithelial and mesenchymal cells [94], and while our studies provide only clues to unravelling the complex environment of the thymus, they do show that CD1a in both skin and thymus is similarly effected by GM-CSF and IL-1 thus supporting a global role for these cytokines in immunoregulation.

It is becoming increasingly clear from our work and that of others that IL-1 and GM-CSF are regulators of immune function associated molecules such as MHC class I and CD1a *in vitro*. Since these cytokines are made by normal human keratinocytes *in vitro* [81,82] it is possible that they play some role in the

regulation of human LC *in vivo*. In addition, IL-1 production has been demonstrated in thymic epithelial cells [88], and GM-CSF production by thymic nonlymphoid cells [89] raising the possibility that these cytokines are also involved in the regulation of antigen expression by thymocytes in the thymus.

While evidence of cytokine production by cells of the skin and thymus supports the conclusion that the phenotypic regulation we have described *in vitro* may be important *in vivo*, this evidence also presents a problem in the interpretation of our data. For example, our LC cultures are typically contaminated with $\geq$50% keratinocytes, and any culture obtained from thymus gland will invariably contain contaminating epithelial cells and fibroblasts. It is known that IL-1 can induce production of GM-CSF in keratinocytes [90], while TNF-α can induce its production in fibroblasts, smooth muscle cells, and endothelial cells [91]. Thus the direct effect of exogenous cytokines may be hard to assess and it may be that our best attempts to create simple *in vitro* systems still result in a complex interaction of cells and signalling molecules, as is the case *in vivo*. Despite these concerns, it seems clear that epidermally associated cytokines influence the expression of immune function associated molecules on human LC and thymocytes. As discussed below, these cytokines also influence the functional abilities of LC *in vitro*.

It has been known for some time that the ability of human Langerhans cells to stimulate allogeneic T-cells increases

dramatically after 2-3 days in culture [71,76], and recent murine studies have shown that GM-CSF can accelerate the development of this ability [69,77]. We have extended this observation using human LC. In particular we have shown that human LC precultured with GM-CSF for 72 hours are 2-3 times more potent than control cells in the stimulation of allogeneic T-cells. While this finding may have been predicted from murine studies, it is important in light of the differences noted between murine and human immune systems. These differences have been demonstrated by the fact that human epidermis lacks a cell equivalent to the Thy 1 positive dendritic T-cells which represent 2-5% of cells in murine epidermis. Since keratinocytes can produce GM-CSF [82], it is possible that the exogenous GM-CSF used in these experiments merely augmented endogenous GM-CSF in the cultures, thus accelerating both the phenotypic and functional changes. Our functional studies with TNF-α and IL-1 showed slightly lower levels of T-cell proliferation than control, however these results were not statistically significant. It is possible that endogenous GM-CSF was subtracting from the magnitude of these cytokines' effects. We are therefore planning blocking studies using anti-GM-CSF antibodies to further isolate the effect of IL-1, TNF-α, and endogenous GM-CSF on LC *in vitro*. It is not clear from our studies whether the increased stimulatory capacity of GM-CSF cultured cells represents a change in LC physiology or is mediated by phenotypic changes such as increased class I MHC or decreased CD1a expression. It is clear that the effect is not due to changes

in viability of LC in culture since viability was the same in all of our experimental and control groups. We were concerned that GM-CSF might cause increased allogeneic T-cell stimulation by a direct effect on T-cells. This is an unlikely explanation for our results for several reasons. For one, LC were carefully washed of cytokine containing medium before addition of allogeneic T-cells. Second, wells in which there was culture medium and GM-CSF but no LC did not show increased T-cell proliferation compared to wells in which there was culture medium, no GM-CSF, and no LC. This finding precludes the possiblity that it was the direct effect of GM-CSF that was solely responsible for the increase in T-cell proliferation seen. It is theoretically possible that a small amount of residual GM-CSF stimulated proliferation of allogeneic T-cells, but since there was probably some endogenous GM-CSF in all cultures, we doubt that it could have had a significant effect.

Another far less likely possibility is that GM-CSF increases the ability of keratinocytes to stimulate allogeneic T-cells. Many studies have shown that depleting LC from epidermal cell suspensions renders these suspensions all but devoid of the ability to stimulate T-cells [50,95]. Studies comparing the ability of injected LC and keratinocytes to prime allogeneic reactions in mice show that LC are $>10^3$ times as potent as keratinocytes at priming allogeneic reactions to subsequent skin allografts [96,97]. It appears then that the ability of keratinocytes to stimulate allogeneic reactions is limited at best, and it is therefore unlikely that they are capable of inducing

the high levels of T-cell proliferation typically associated with LC, even under the influence of GM-CSF.

It is reasonable to say then that the increased stimulation seen in our GM-CSF treated cultures is probably due to a direct effect on LC. The mechanism of this effect has not been discerned in either human or murine systems. It is attractive to postulate that this increased functional ability is in some way related to the changes in surface phenotype associated with GM-CSF pretreatment, and our blocking studies are an attempt to explore this possibility.

In these blocking experiments all LC used were pretreated with GM-CSF in order to compare the effects of the three blocking monoclonal antibodies used (anti-class I MHC, anti-class II MHC, and anti-CD1a) to GM-CSF treated LC alone. As predicted from many *in vitro* and *in vivo* studies of antigen presentation by LC, anti-class II MHC blocked T-cell stimulation in a statistically significant way, even though the effect was saturated at higher doses of LC. The anti-class II MHC antibody used in these cultures was a filtered, titered hybridoma supernatant, and the precise concentration of antibody was unknown. It is possible, therefore, that high numbers of LC overcame the class II MHC blockade, since the amount of anti-class II MHC supernatant used was constant throughout our cultures.

An interesting and unpredicted finding from these blocking experiments is the significant decrease in T-cell stimulation seen in anti-class I MHC treated cultures at all doses of LC

tested. This is a novel observation in MELR's using LC, and we are unaware of other workers in animal or human systems reporting or even attempting this type of blockade. It is possible that the anti-class I MHC antibody added to cultures is producing this blockade in an idiosyncratic or nonspecific fashion, however the other more interesting possibility is that LC *in vitro* are involved in allogeneic T-cell stimulation in a non-class II MHC restricted fashion. There is some indirect evidence to support this notion in the findings of Romani et al which show that human LC dramatically increase their expression of MHC class I surface antigens after 2 to 4 days *in vitro*, while increasing their ability to stimulate allogeneic reactions [71].

Finally, the addition of anti-CD1a monclonal antibody had no effect on the levels of T-cell stimulation seen when comapared to control cultures without blocking antibodies. CD1a has many similarities to MHC class I molecules as discussed above [26-35], and it has been speculated that CD1a may then have a function similar to class I MHC molecules, namely antigen presentation to CD8 positive T-cells [57,97]. $CD8^+$ T-cells are generally associated with suppressor and cytotoxic functions [54], and their proliferative response to stimulation is generally smaller and shorter in duration than that of $CD4^+$ cells [99]. $CD8^+$ cells are also difficult to grow because they often kill the cells that stimulate them [100], and have stringent growth requirements including the presence of IL-2 [101]. As a result, stimulation of $CD8^+$ cells by LC bearing CD1a could easily be overlooked in

MELR's containing large numbers of $CD4^+$ cells. If such stimulation were obscured, blockade of stimulation would surely be obscured as well. Thus, the lack of blockade of T-cell stimulation by anti-CD1a antibody in our cultures does not preclude the possibility that CD1a is functioning as an antigen presenting molecule for a subset of $CD8^+$ lymphocytes.

In order to explore the possibility of class I MHC (or CD1a) stimulation of $CD8^+$ cells *in vitro*, we are planning a series of experiments using GM-CSF cultured LC and fractionated $CD4^+$ and $CD8^+$ subsets of T-cells in MELR's. In addition, these experiments will use IL-2 in T-cell culture medium in order to amplify the proliferative response of $CD8^+$ cells [101]. These experiments may shed additional light on the possibility of LC stimulating allogeneic T-cells in a non-class II MHC restricted fashion. Early results from these experiments indicate that LC can induce proliferation in allogeneic $CD8^+$ T-cells. This finding is a very important piece of evidence in proving that LC stimulate the immune system through circuits other than the MHC class II/$CD4^+$ T-cell pathway. However, more work is required to confirm this very preliminary finding. There is some evidence that LC present antigen in a non-MHC class II restricted fashion in a recent study by Porcelli et al. In this study, the authors demonstrate the specific recognition of CD1a by a $CD4^-/CD8^-$ (double negative) cytotoxic T-cell line which causes direct lysis of CD1a bearing cells [102]. Furhtermore, this lysis can be blocked with anti-CD1a monoclonal antibodies [102]. The CD1a positive cells used

in this study, however, were the MOLT-4 thymic leukemia cell line and thus proof of non-MHC class II restricted antigen presentation by physiologic LC is still lacking. While a great deal of work is still needed to understand the regulation of LC and their unique surface antigen CD1a, work like ours and Porcelli's has begun to describe the regulation of the observed phenotype and function of LC, and may eventually lead to a precise understanding of the role of these cells in the immunology of the skin.

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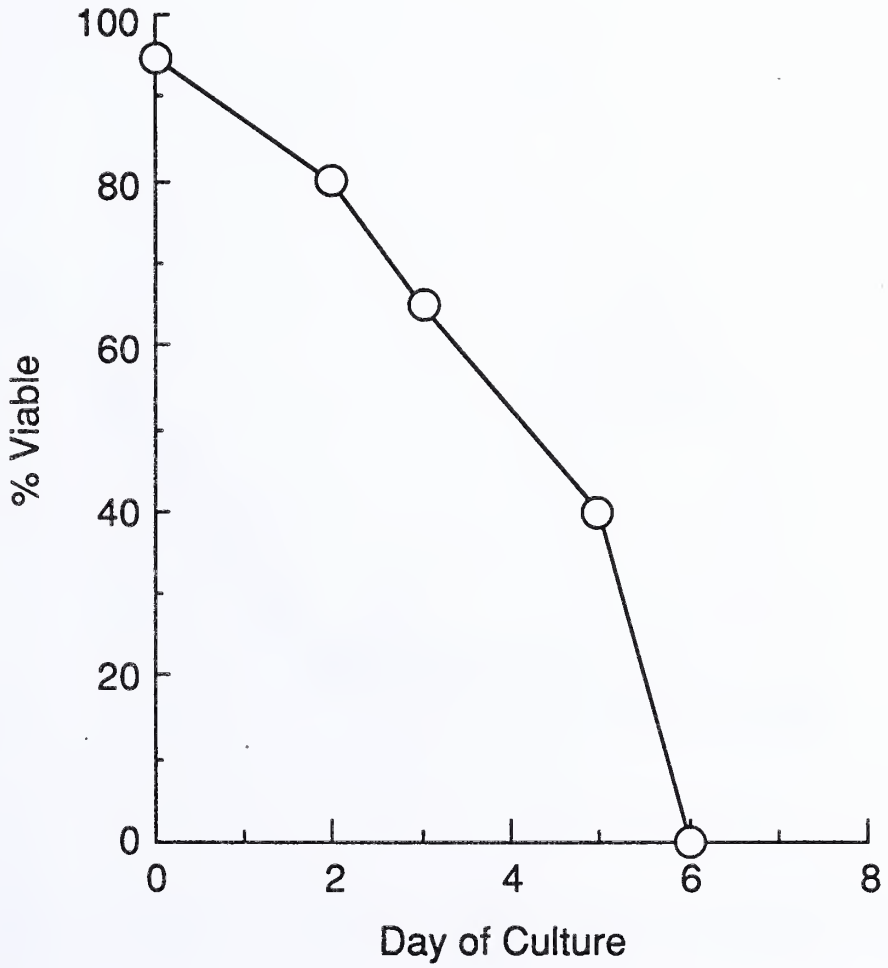

Figure 1. viability of Langerhans cells *in vitro* as assessed on days 0, 2, 3, 5, and 6 of culture by trypan blue exclusion. There was no difference in viability in cultures treated with IL-1, GM-CSF, TNF-α, or control.

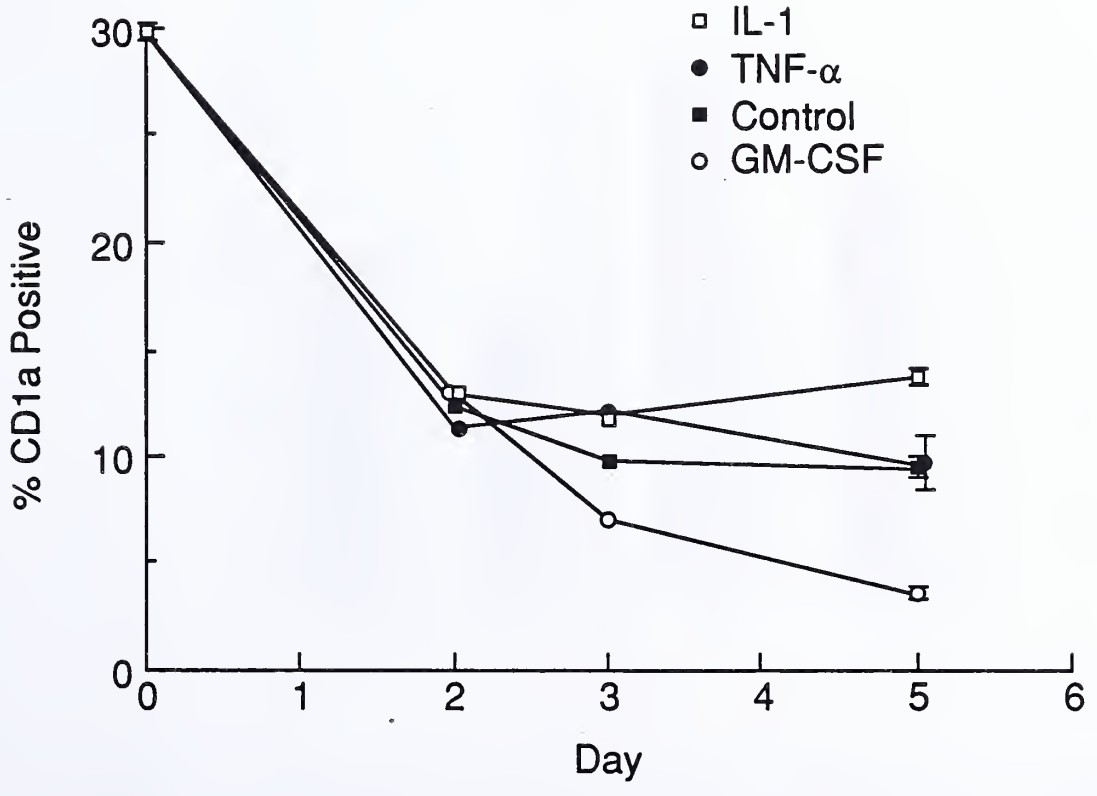

Figure 2. percentage of cells expressing CD1a surface antigen as assessed by flow cytometry on days 0, 2, 3, and 5 of culture. By day 5 control cells were 9.75±1.2% CD1a positive, while GM-CSF treated cells expressed significantly lower levels of CD1a (4.2±0.26%, p=0.01), and IL-1 treated cells expressed significantly more CD1a (14.45±0.04%, p=0.02). TNF-α did not produce a significant change in CD1a expression (11.35±1.2%, p=0.5).

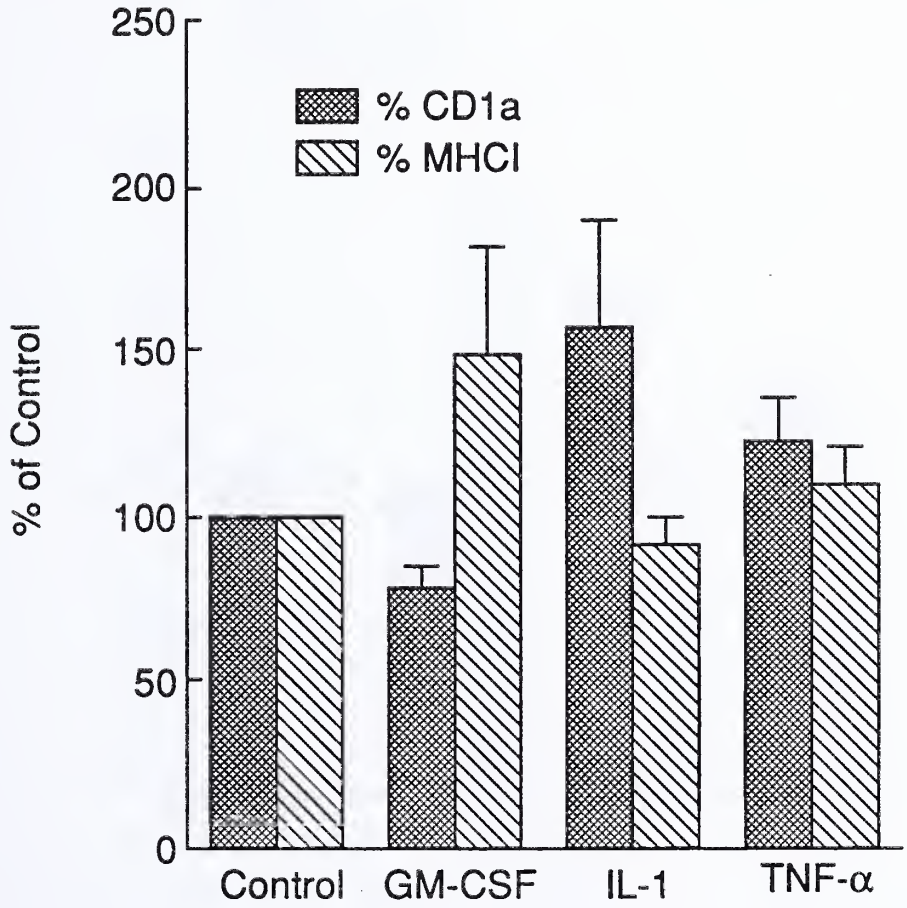

Figure 3. Undepleted thymocyte cultures. GM-CSF was associated with decreased CD1a expression (78±7.4%, p=0.03), and increased MHC I expression (148±34%, p=0.02). IL-1 was associated with increased CD1a expression (157±33%, p=0.01), while all other effects were satistically insignificant.

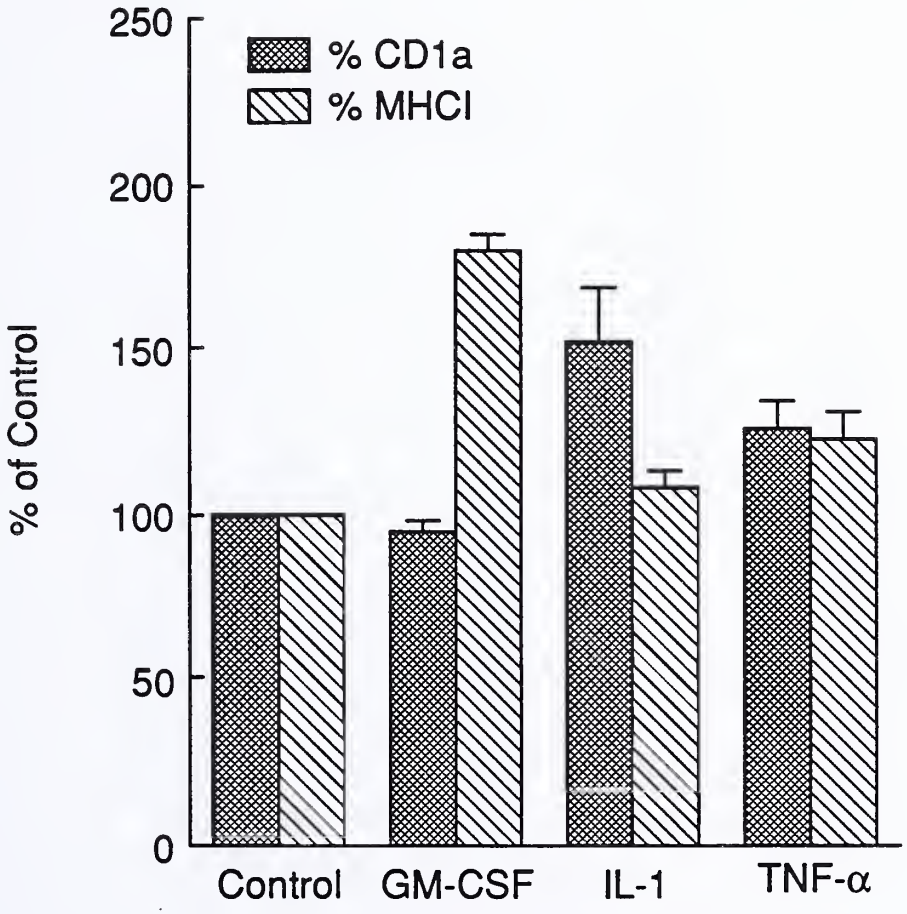

Figure 4. MHC I depleted thymocyte cultures. GM-CSF was associated with increased MHC I expression (180±4.0%, p=0.008), and IL-1 with increased CD1a expression (152±17%, p=0.03). All other effects were insignificant.

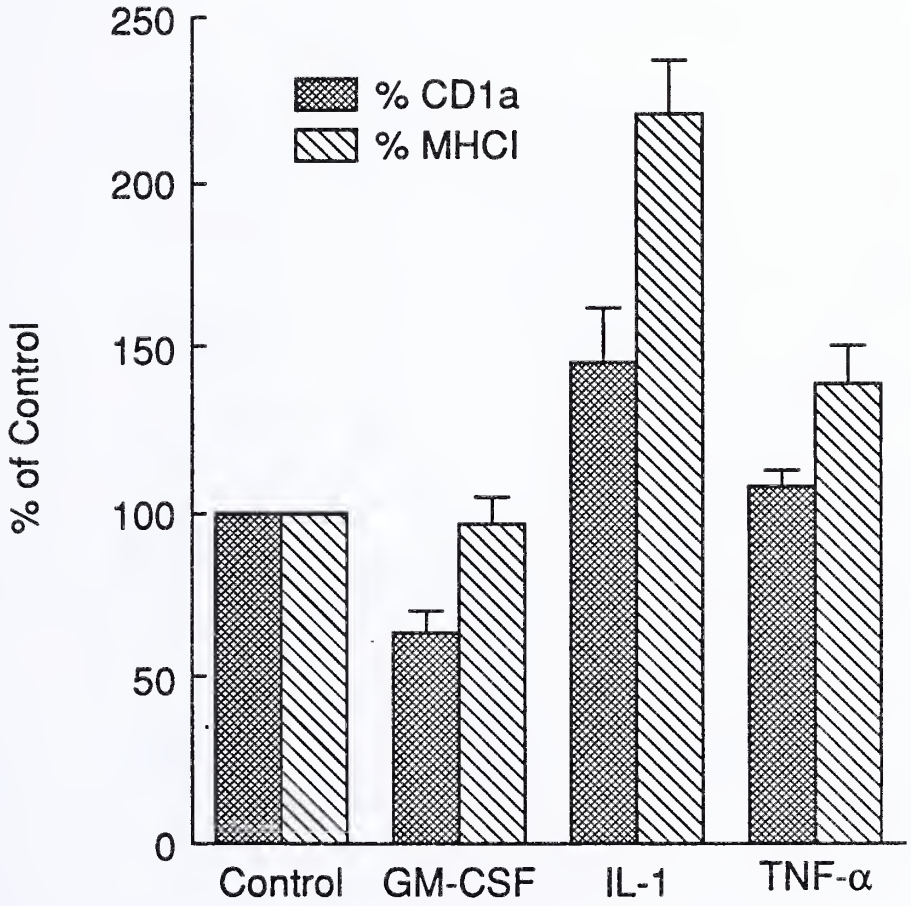

Figure 5. CD1a depleted thymocyte cultures. GM-CSF was associated with decreased CD1a expression (64±6%, p=0.01). IL-1 was associated with increased CD1a expression (146±16%, p=0.03), and increased MHC I expression (220±17%, p=0.08). All other effects were insignificant.

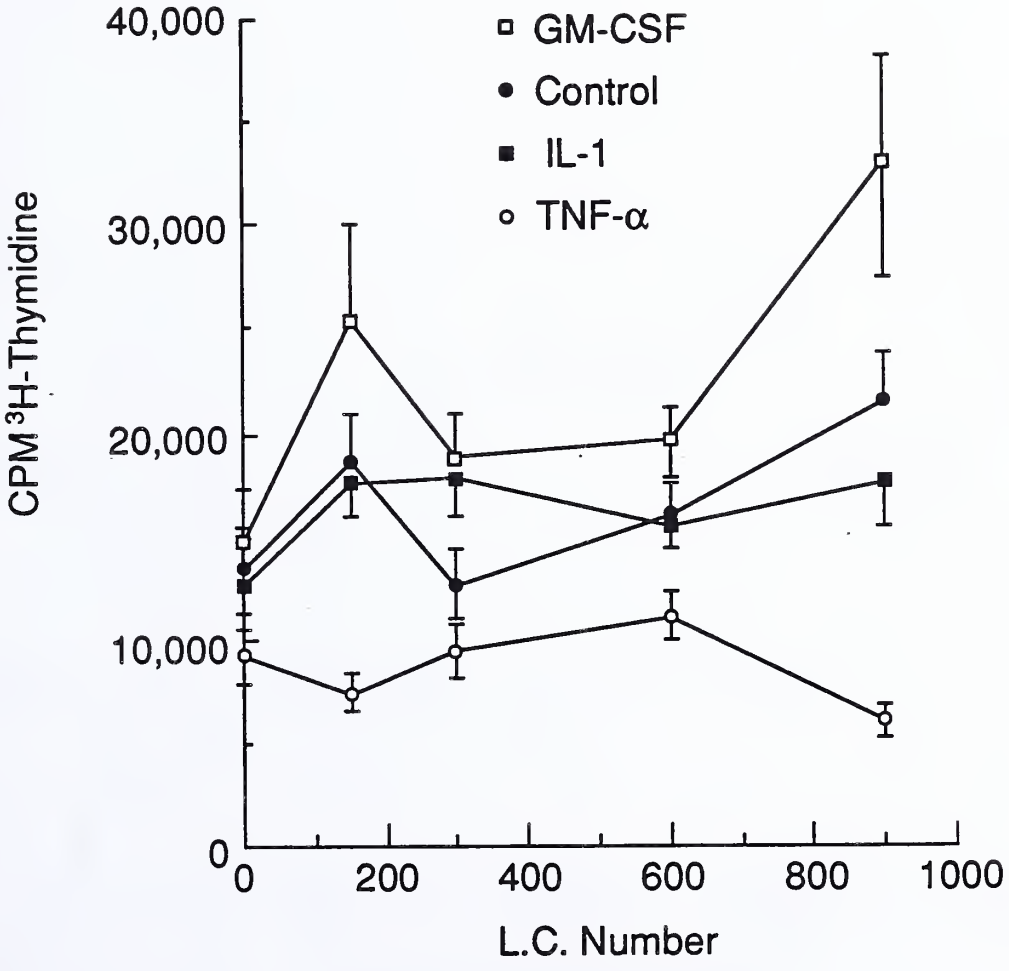

Figure 6. Langerhans cell/T-cell MELR. LC pretreated with IL-1 produced levels of T-cell proliferation unchanged from control, as did LC pretreated with TNF-α (except at 900 LC where TNF-α produced significantly less T-cell proliferation than control $p=0.05$). GM-CSF pretreatment produced significantly increased levels of T-cell proliferation ($p \leq 0.02$) except at 300 LC ($p=0.5$).

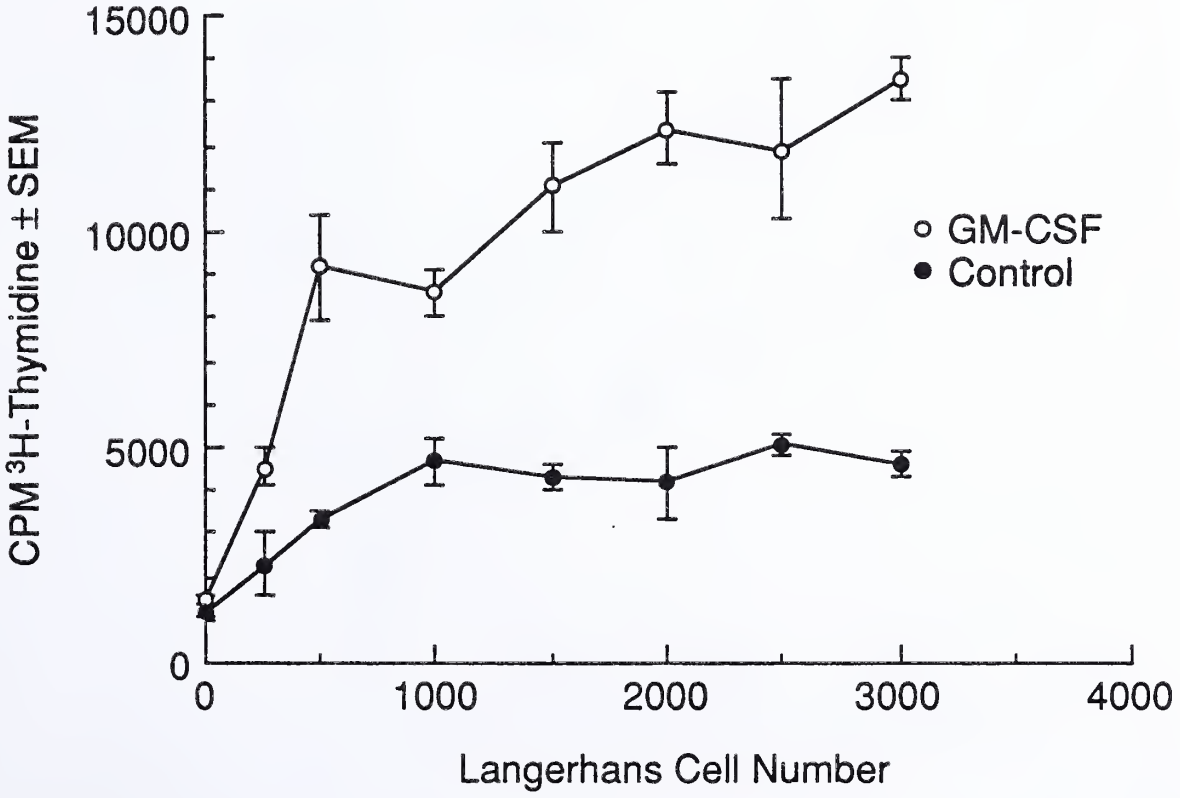

Figure 7. Langerhans cell/T-cell MELR focusing on GM-CSF. GM-CSF produced increased levels of T-cell proliferation at $p \leq 0.01$ at all doses of LC tested except 250 where $p=0.05$. In wells in which there were no LC there was no difference in the amount of T-cell proliferation seen in control versus GM-CSF treated cultures ($p=0.3$).

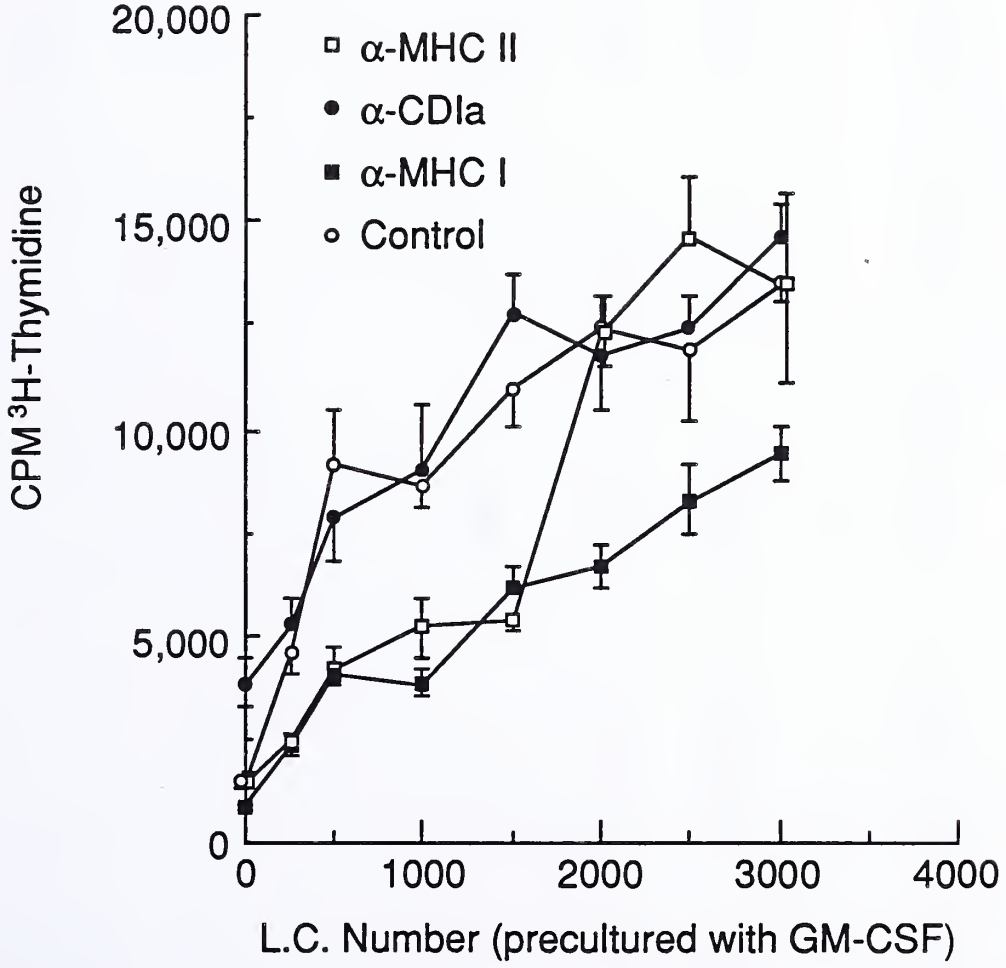

Figure 8. Langerhans cell/T-cell MELR in which all LC were pretreated with GM-CSF. At the time of T-cell addition, blocking antibodies to either MHC I, MHC II, or CD1a were added to culture. Anti-MHC II produced a significant reduction in T-cell proliferation at 0-1500 LC ($p \leq 0.03$), but no reduction at 2000-3000 LC ($p=0.5$). Anti-MHC I produced a significant reduction at all doses of LC ($p \leq 0.03$). Anti-CD1a did not produce a significant change in T-cell proliferation compared to control ($p=0.5$).

	Effect on MHC I Expression			Effect on CD1a Expression		
	Undepleted	CD1a Dep.	MHC I Dep	Undepleted	CD1a Dep	MHC I Dep
Cytokine						
GM-CSF	increase	no effect	increase	decrease	decrease	no effect
IL-1	no effect	increase	no effect	increase	increase	increase
TNF-α	no effect	no effect	no effect	no effect	no effect	no effect

Table 1. table 1 summarizes the significant effects of IL-1 and GM-CSF on all thymocyte cultures. TNF-α failed to produce any significant effects.

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