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Examination of Estimated 24 Hour Period of Dendritic Cell Maturation

Sarah Reeves

Senior Research Project

Advisor: Dr. Ann Foster

4/18/03

Abstract:

Dendritic cells (DCs) are responsible for antigen uptake, presentation, and stimulation of T cells responsible for recognizing these antigens. During the immature stage DCs endocytose fluids and particles from the surrounding interstitial environment, such as the skin, deeper organs, and mucous membranes. Maturity is reached after antigen uptake stimulates the loading of foreign peptides on their major histocompatibility complex class II (MHC II) molecules, which then are expressed on the cell surface. DCs cease antigen uptake and presentation at some point after the uptake of a maturation stimulus (antigen); at this point the DC is fully mature. It has been estimated that these cells have a 24 hour maturation period. However, recent experiments suggest that this period is longer than 24 hours.

Introduction:

Dendritic cells (also called Langerhans cells) are found in the peripheral fluid of organs and mucous membranes (Funnel 2002). DCs develop from cells known as nonproliferating precursors, which in turn have evolved from proliferating progenitor cells, which are located mainly in the bone marrow (Steinman et al. 1999).

A DC is characterized by its stellate or dendritic (branching) shape (Fig 1). It has a dark nucleus and clear cytoplasm, within which can be found Birbeck granules, whose function are

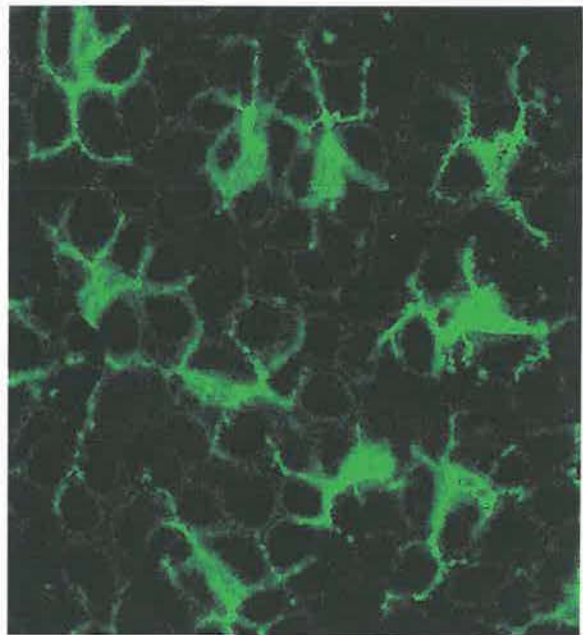


Fig 1 These fluorescently-tagged DCs exemplify the branching, "dendritic" nature of this type of cell (Mellman et al. 1998).

currently under study(Funnel 2002). In the immature stage, a DC circulates throughout tissues and lymphoid organs, endocytosing material such as self antigens and proteins from its surroundings (Steinman and Nuzzen 2001). An immature DC is stimulated to begin maturation upon endocytosis of a stimulating antigen, (a foreign substance) such as bacterial peptides. Stimulated epidermal DCs leave the skin and migrate to draining lymph nodes via lymphatic vessels upon bacterial invasion (Shartman 2000) where they initiate an immune response by stimulating cytotoxic T lymphocytes (Morrow 2001). Major Histocompatibility Complex (MHC) class I and class II molecules are used by DCs to capture and present peptides to T-cells. Class I molecules predominantly bind peptides from endogenously synthesized cytosolic proteins and class II molecules present peptides derived from internalized degraded material (Nordeng et al. 1996). During the intermediate stage of maturation, the DC processes and loads the antigen's peptides onto its MHC II molecules and transports them to the cell surface (Mellman et al. 1998). Once the DC has completed its task of processing and loading large numbers of the antigen peptides it is considered a mature cell (Banchereau and Steinman 1998) like the one in Figure 2. In this mature state, the peptides presented on its MHC II loaded membrane can be found and

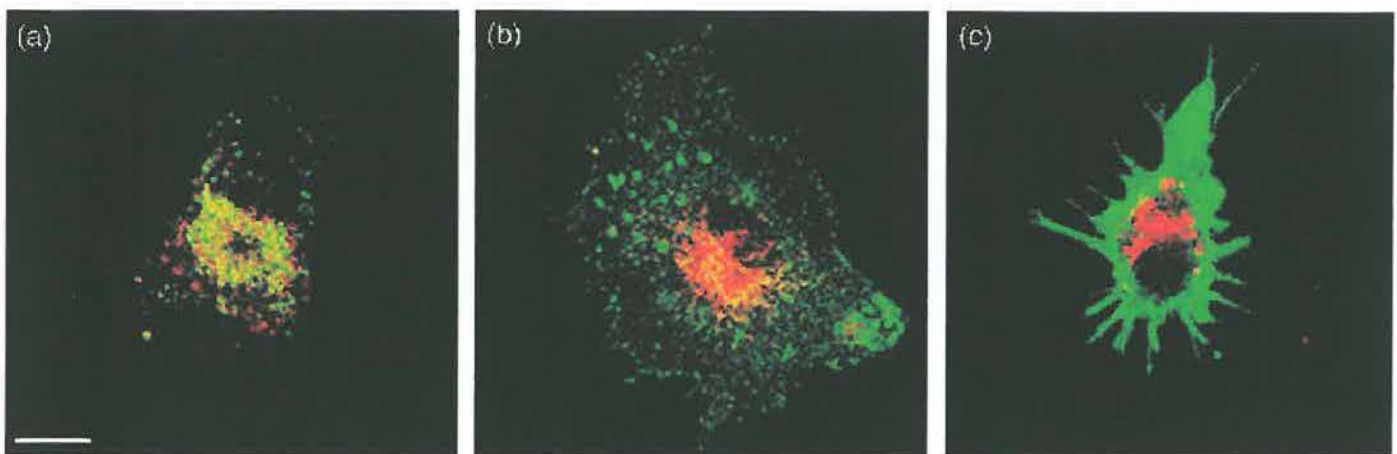


Fig 2: As dendritic cells mature, antigens are loaded onto MHC II molecules and sent to the surface of the membrane for presentation. In these pictures, MHC II molecules are stained green, and can be seen to emerge on the membrane as the cell goes from (a) the immature state to (b) the intermediate state, and finally to (c) the mature state (Mellman et al. 2001).

recognized by lymph node T cells. The branched shape of the DC provides more surface area for presentation of peptide to T cells (Mellman and Steinman 2001).

Each helper T cell (T_H) has its own specific T cell receptor designed for a single ligand. Helper T cells are attracted by the chemokines released by the DC and are activated when they encounter this specific combination of MHC II and antigen peptide (Cyster et al. 2000). Once stimulated, the bound T cell releases interleukin-2 (IL-2) to stimulate other T cells in the vicinity, initiating a larger immune system response.

The time period between initial stimulation and complete maturation has not been determined, but has been estimated to be about 24 hours (Inaba et al. 1992). The purpose of this study is to determine the maturation time for an antigen stimulated DC. Pigeon cytochrome C (PCC) is used as the antigen. The cessation of endocytosis acts as the indicator of maturation (Mellman and Steinman 2001).

Dendritic cell study is spread throughout many areas of medical research. Specialized DC therapy may play a role in preventing post-donation organ rejection by interacting with donor-reactive T cells to minimize rejection side effects (Takayama et al. 1999). DCs may play a role in vaccines against cancer (Curti et al. 2001). Researchers hope to use DCs to fight tumors by isolating patient DCs, culturing/priming them with tumor antigen, and then vaccinating the patient with their "educated" DCs (Skin Cancer Foundation 2002). DCs may someday help HIV, AIDS, and other patients with immunosuppressive conditions, battle infections against opportunistic fungi. Success has already been achieved against the fungus *Pneumocystis carinii* (Ashkenas 2001). Research is underway for using DCs to vaccinate against multiple myeloma (Yi et al. 2001). DCs could someday help prevent or treat diabetes. Specially engineered DCs may be used to destroy T-cells which are killing the host's insulin-producing cells (Rossi 2002). However,

much research remains to be conducted before these procedures can be optimized. This is why further research on the details of DC behavior, such as maturation timing, is so badly needed.

Materials and Methods:

Cell Line:

In these experiments AND3 hybridoma T cells were used to recognize I-E^k MHC class II molecules expressing PCC⁹⁸⁻¹⁰⁴ (the segment of processed PCC peptide containing amino acids 98 through 104) on the cell membrane. Dendritic cell strain C3D2F1 was used because it could present I-E^k MHC class II molecules. These DCs therefore had the potential of being fed PCC and presenting the peptides on I-E^k MHC II molecules in a manner that could stimulate AND3 T cells to release IL-2. DCs were isolated in the lab from mouse bone marrow, following lab protocol by Inaba et al(1992). T_H cells were hybridomas (modified T cells that were fused with AND3 lymphoma cells)(Pharmingen) to ensure cell immortality. T cell hybridomas were cultured in RPMI (a culture media named after the Roswell Park Memorial Institute)1640 (Gibco) with 10% heat-inactivated FCS (fetal calf serum), 2mM L-glutamine (Gibco), 1mM sodium pyruvate (Gibco), 1 mM non-essential amino acids (Gibco), 0.75 mM essential amino acids (Gibco), 50 mM BME (basal medium eagle's) (Sigma), 0.075 glucose (Gibco), and 10mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (Gibco). Cells were incubated at 5% CO₂, 37°C. Cells were given fresh media every other day.

Cell thawing:

Dendritic cells had been frozen for storage in 1.0 ml vials containing approximately 1×10^7 dendritic cells. Cell vials were removed from liquid nitrogen and placed immediately on ice, to prevent shock during their transition from the storage room to the water bath area. Vials were shaken in a 37°C water bath until all but a small ice pellet thawed. Cells were then placed in cold

growth media, allowed to sit on ice for 5 minutes, then centrifuged at 1200 rpm for 5 minutes. After aspirating the media, cells were re-suspended at a concentration of 2×10^6 by first tapping in residual media and then adding warmed growth media.

Antigen Presentation:

Antigen presentation assays were done by exposing post-maturation-stimulated DCs to antigen molecules at intervals between 0 and 24+ hours and washing out antigen three hours later following preparation, buffering, and concentrations as described in the antigen presentation laboratory protocol by Buus et al(1986). Intervals varied for each assay; the first covered from 2 to 10 hours after exposure, the second from 14 to 28 hours, the third from 24 to 36 hours, and the fourth from 6 to 20 hours. DCs were first given LPS (lipopolysaccharide: a bacterial membrane component extracted from *L. monocytogenes*), or ovalbumin as their maturation stimulus. These samples were then each pulsed with PCC, (which also contains LPS) at intervals between 0 and 36 hours. T cells responsible for recognizing antigen-loaded MHC IIs were immediately added to DCs at a 1:1 ratio and incubated at 37°C, 5% CO₂ for 24 hours. In this experiment, presentation of PCC was tested by antigen presentation assays using the AND3 hybridoma T cells, which released IL-2 when stimulated by contact with presented PCC peptides. Levels of hybridoma activation were measured by averaging anti-IL2 ELISA concentrations from all the wells of each time period or standard, a total which represented the antigen presentation of approximately 90 DCs per group.

Cluster disruption technique:

In later experiments it became necessary to ensure that all DCs had truly received a maturation stimulus at the same time. As an additional maturation stimulus, a P1000 pipet was used to triturate DC samples ten times three hours after the addition of antigen. This disturbance

stimulated the cells to enter the intermediate phase without antigen uptake.

ELISA:

Twenty-four-hour supernatants from antigen presentation (AP) assays were collected by micropipet and transferred to fresh plates after centrifugation of the original plates at 1500 RPM for 5 minutes. Supernatants were frozen at -80°C overnight before use. Anti-IL-2 ELISA protocol was followed according to manufacturer instructions (Pharmingen) to measure T cell stimulation. Plates were read with an optical density of 450 nm.

Antigens/reagents:

Anti-CD3, anti-CD28, anti-IL-2 (biotinylated and unconjugated) were obtained from Pharmingen; PMA (phorbol-myristate-acetate), calcium ionophore, ovalbumin, PCC, and LPS were purchased from Sigma. Streptavidin-horseradish peroxidase was from Zymed.

Controls:

Each experiment's plate contained wells with several positive and negative controls for both dendritic cells and T cells. Positive DC controls included maturation only by PCC to ensure that these DCs were indeed capable of processing entire PCC molecules, presenting the processed peptides, and stimulating the proper T cells. Mature DC surface MHC II molecules were loaded with MCC-peptide (moth cytochrome c-peptide: differing from PCC-peptide in only one amino acid) to ensure that pre-processed peptides had no trouble being loaded and presented on MHC II molecules. Positive T cell controls included exposing T cell wells to PMA + calcium ionophore (P/I) and stimulating other wells with antibodies recognizing the T cell receptors CD3 and CD28 (costimulatory molecules also necessary for successful T cell activation). Negative controls included samples of DCs + LPS (without PCC), T cells only, and DCs only.

Results:

Because the supernatants of the assays were diluted at various ratios, each was judged by its own respective scale (set by the standards and controls used on each plate) to measure IL-2 levels, and was not compared with the scales of the other graphs. Ratios varied due to the number of DCs used in each assay, each chosen to best fit the respective amount of IL-2 that was going to be measured. The more cells that were available for the assay, the higher the dilution of the supernatants to avoid oversaturation. The ratios were not always a perfect match, however, resulting in some variance in scales between the graphs. Assays two and four had a dilution of 1:1, so their IL-2 values fell between 1 and 2 ng/ml. Assay three had a dilution of 1:5, and had a range of IL-2 values between 1.5 and 3 ng/ml. Assay one had a dilution of 1:2, and had a range of IL-2 levels between 1.5 and 4.5 ng/ml.

AP assay 1 involved pulsing dendritic cells at time points between 2 and 10 hours after the maturation stimulus (LPS) was given. In the samples of PCC-pulsed DCs + AND3 T cells, a rising titer was observed between these time points (Figure 3). As the time between stimulation

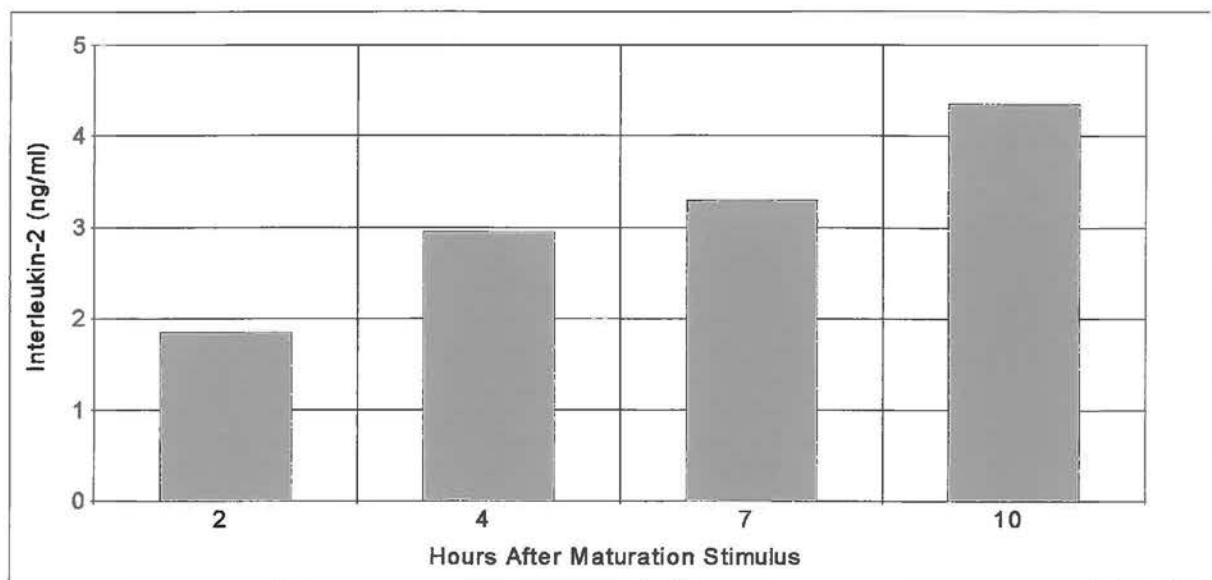


Figure 3: T cells reacting with DCs that were pulsed with PCC up to ten hours after receiving a maturation stimulus show a rising titer of IL-2.

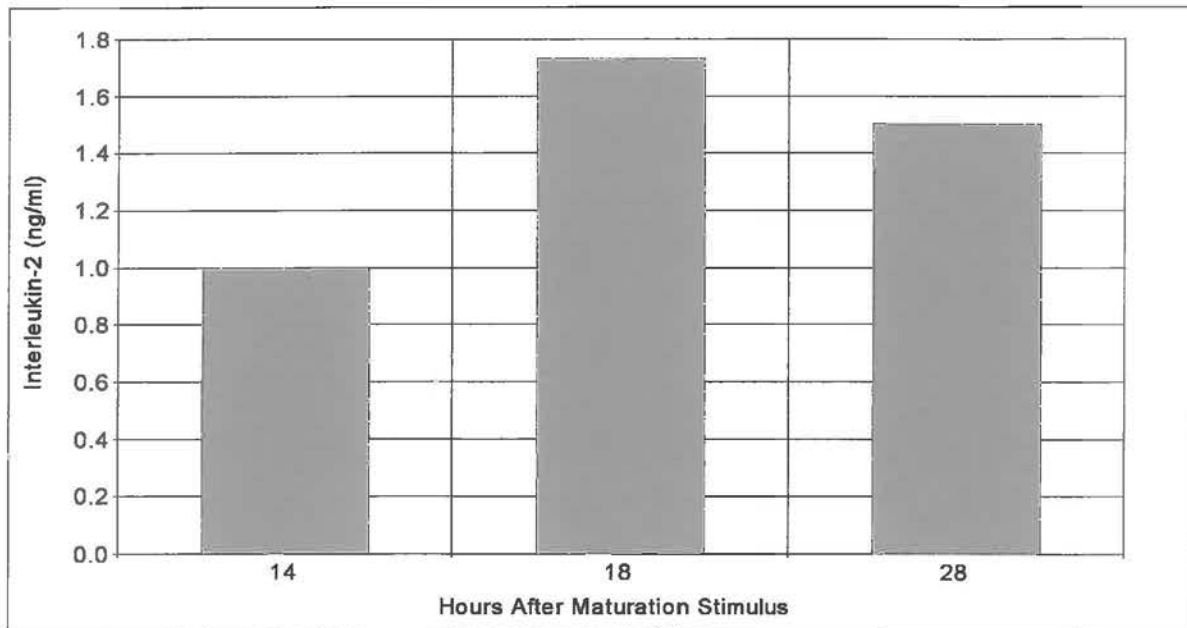


Fig 4: T cells reacting with DCs that were pulsed with PCC up to 28 hours after receiving maturation stimulus still show high levels of IL-2.

and antigen introduction increased, so did the amount of interleukin-2 released by the T cells in response to increased antigen presentation on the dendritic cells' surfaces. IL-2 levels increased approximately 2.5 times between the first and final readings.

AP assay 2 dealt with time points after 10 hours, measuring antigen presentation up to 28 hours after the introduction of the maturation stimulus (Figure 4). AND3 T cells continued to show significant levels of activation up to the 28 hour time point. There was a small drop in IL-2 levels between the 18 and 28 hour time periods. However, because this drop was small and because this 18 hour level of IL-2 still fell within acceptable IL-2 levels set by the positive controls on that plate, the IL-2 levels at 28 hours still indicated actively endocytosing and antigen-presenting dendritic cells.

At this point in the experiment, it became necessary to examine the possibility that our previous results were misleading. Because DCs tend to form clusters along the sides and bottom

of the wells as they multiply, it could have been possible that the cells inside the clusters were receiving the maturation stimulus later than those outside or were hiding DC precursors, making it appear that maturation took longer than it really did. Since these inner cells would take longer to mature than those outside the cluster, they could have given the illusion that DC uptake continued past 24 hours. To test the reliability of our previous results, the cluster disruption technique (see materials and methods) was employed on AP assay 3. An ELISA of this assay indicated antigen presentation up to 36 hours after the initial PCC stimulation (Figure 5). There was a slight drop in IL-2 at the 29 hour period, but it still fell within acceptable IL-2 levels set by the positive controls on that plate.

Because the maturation period still appeared to be extending much farther than the estimated 24 hour period, further measures were taken to ensure that all the dendritic cells received the maturation stimulus at the same time, since it was believed that clusters of cells could reform following disruption. Since dendritic cells break away from their clusters when they

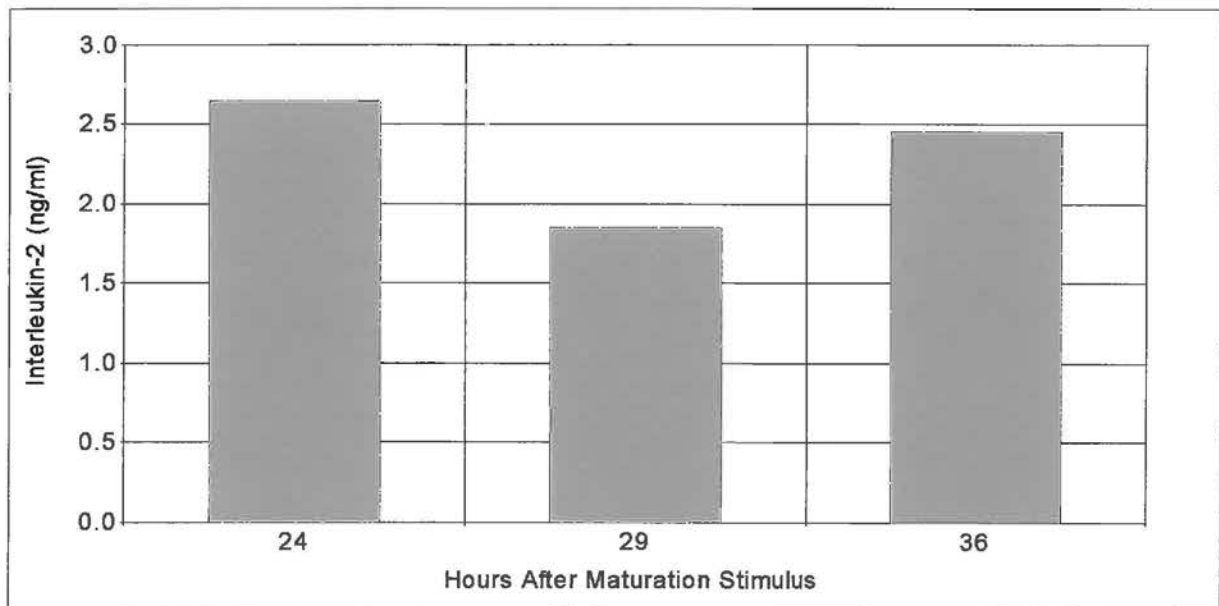


Figure 5: T cells reacting with DCS that were pulsed with PCC up to 36 hours after receiving maturation stimulus still show high levels of IL-2.

receive a stimulus, AP assay 4 was conducted using only free floating dendritic cells; those clustering around the walls of the wells were discarded since it was not certain if all of them received the maturation stimulus (LPS) at the same time. Up to 20 hours after stimulation the DCs were still presenting antigen and showed no decline of IL-2 levels (Figure 6). However, the drop in the 10 hour period is a significant drop, but why it happened could not be determined. It is probably due to some unknown experimental error stemming from the care or handling of this sample of cells.

Due to time restraints, post-20 hour experimentation with free floating cells was not able to be explored.

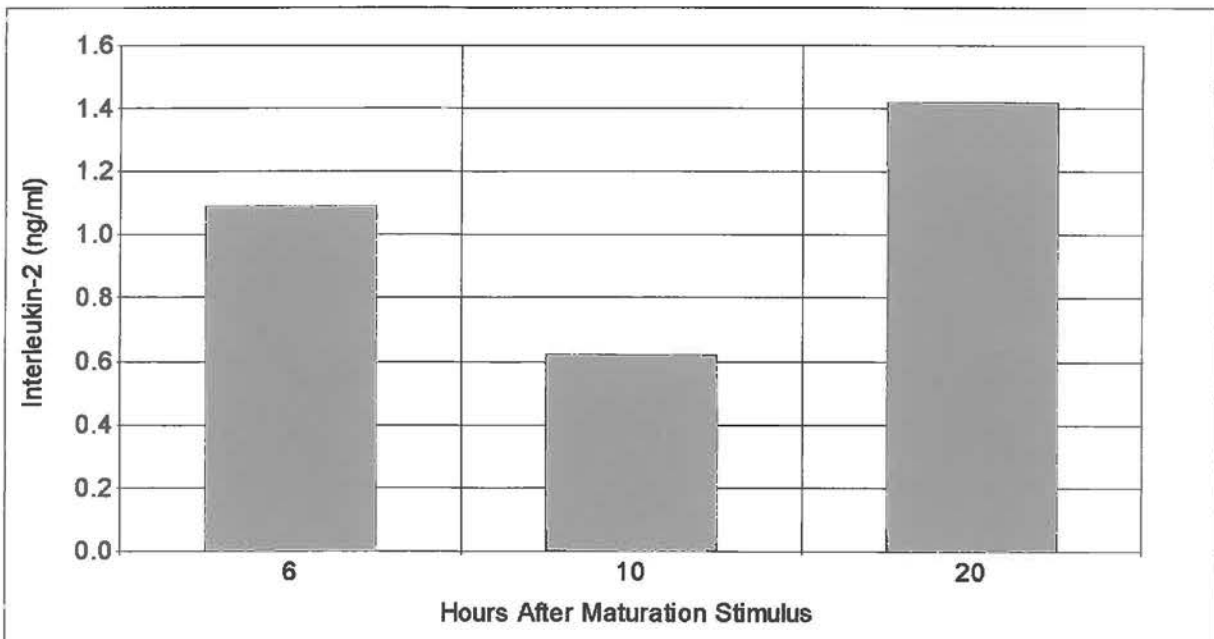


Figure 6: IL-2 levels show no overall decline from T cells reacted with free floating DCs.

Discussion:

The overall results from the antigen presentation assays indicate that dendritic cell maturation may take longer than the estimated 24 hour period. The first three experiments showed high levels of interleukin-2 past 24 hours, even up to 36 hours in AP assay 3, with no sign of decline. Except for slight IL-2 variations still within levels set by positive controls in AP assays 2 and 3, or the one large drop in IL-2 in AP assay 4 due to experimental error, there was no telltale drop in IL-2 levels that would indicate that dendritic cell endocytosis had ceased.

However, because of time constraints and many other AP assays that were lost due to various experimental errors or faulty materials, research concerning the clustering effect of DCs on these experiments was not completed. Because there was only time to test maturation with free floating cells up through 10 hours, it cannot be said with certainty that DCs take longer than 24 hours to mature.

Future research should focus on repeating these experiments to verify their results, testing this hypothesis that the maturation of DCs requires longer than 24 hours. Future research should explore the possibility that DC precursors were present throughout the experiments and influencing the IL-2 results. To do this researchers would need to separate precursors from DC samples. This can be done by coating metallic beads with antibodies to CD11c (a “marker molecule” only present on DCs), exposing DC solutions to these beads, and placing the cells over a magnetic column to extract only DCs and yield a pure population.

The effect of antigen concentration in maturing DCs also needs to be further studied. It may be that the concentrations used in these experiments were too high, saturating the DCs with antigen, which may have led to the appearance of antigen uptake and presentation past the true point of maturation.

Acknowledgments:

First and foremost, my thanks go to my lab supervisor, Juli Unternaehrer. Without her generous gift of time and effort, this project would never have existed let alone developed as far as it did. She was always extremely patient with my stream of novice questions, problems, and lab errors. She demonstrated and ran many of the procedures for me until I became experienced enough in the necessary laboratory techniques. She often kept me from what could have been many late, lonely hours in the lab by offering to take care of long, late night pulsing schedules. On days when I was discouraged by my inadequate lab knowledge or poor ELISA results, she always offered words of encouragement. Juli, thank you so much!

I also wish to thank Ira Mellman of Yale Immunology Labs for allowing me the privilege of joining his laboratory for the summer and being able to learn as much as I have.

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SOUTHERN SCHOLARS SENIOR PROJECT

Name: Sarah Reeves Date: 8/31/01 Major: Biology

SENIOR PROJECT

A significant scholarly project, involving research, writing, or special performance, appropriate to the major in question, is ordinarily completed the senior year. The project is expected to be of sufficiently high quality to warrant a grade of A and to justify public presentation.

Under the guidance of a faculty advisor, the Senior Project should be an original work, should use primary sources when applicable, should have a table of contents and works cited page, should give convincing evidence to support a strong thesis, and should use the methods and writing style appropriate to the discipline.

The completed project, to be turned in in duplicate, must be approved by the Honors Committee in consultation with the student's supervising professor three weeks prior to graduation. Please include the advisor's name on the title page. The 2-3 hours of credit for this project is done as directed study or in a research class.

Keeping in mind the above senior project description, please describe in as much detail as you can the project you will undertake. You may attach a separate sheet if you wish:

see attached

Signature of faculty advisor *SA [unclear] for Ann Foster (on Submittal)* Expected date of completion *May 2001*

Approval to be signed by faculty advisor when completed:

This project has been completed as planned: *Yes LAT*

This in an "A" project: *Yes LAT*

This project is worth 2-3 hours of credit: *Yes LAT*

Advisor's Final Signature *Linda Ann Foster*

Chair, Honors Committee _____ Date Approved: _____

Dear Advisor, please write your final evaluation on the project on the reverse side of this page. Comment on the characteristics that make this "A" quality work.

(won't graduate until May 2001)

DC Proposal

Introduction/Background:

Dendritic cells (DCs) are a crucial participant in the humoral immune system. Their primary function is screening body tissue/fluids for foreign antigens, and, if an antigen is found, activating the proper T cells programmed to respond to that specific antigen.

Early, or immature DCs are found throughout the periphery of the body, such as the skin, where "enemy" organisms can easily enter and infect the body. It is in this stage that a DC is most active in endocytosing particles in its surrounding environment. These particles are transported to lysosomes within the DC. Here, the particles are degraded to simpler peptide molecules. These peptides (or antigens), if of foreign origin (such as a virus, bacteria, etc), will alert the DC to begin maturation.

The DC now leaves the periphery of the body (skin) and enters the lymphatic system, making its way toward the lymph nodes. At this intermediate stage, the cell begins to load the "enemy" peptides onto its MHC (major histocompatibility) II molecules and to transport them to the surface of the cell, the plasma membrane. The point in the intermediate phase where the DC stops endocytosis and begins costimulatory action has not yet been determined.

Once within the lymph nodes, the DC has now entered the late or mature phase. Endocytosis has dramatically decreased at this point. Displaying the peptides on its membrane surface, the mature DC is able to activate the peptide-specific T cells upon contact. An early indication of T cell activation is its production of interleukin-2 (IL-2).

Proposal:

In studying these processes, I seek to answer two questions. First, how many hours after

the initiation of maturation will a DC continue to actively endocytose and present foreign antigens? Second, is it possible for a DC to successfully uptake and display two different antigens such that it will then successfully stimulate both respective T cells?

Procedures:

The DC cells under study are from strain, C3D2F1, selected specifically because they present both I-E^k and I-A^d classes of MHC II molecules. Ovalbumin and PCC act as the two antigens in this experiment. NFAT and AND3 act as their respective T cells, AND3 recognizing I-E^k + PCC⁸⁹⁻¹⁰⁴, and NFAT recognizing I-A^d + Ova³²³⁻³³⁹ (where the superscripts 89 - 104 and 323 - 339 refer to the specific amino acid sequences taken from the antigen and loaded onto the MHC II molecules).

In a series of antigen presentation assays, DCs will be initially stimulated by LPS (for single antigen uptake study), or ovalbumin or PCC (for double antigen uptake study). These samples will be individually pulsed with the second antigen after 2 hrs, 4 hrs, 6 hrs, 8 hrs, etc. up to at least 24 hrs. To distinguish between AND3 and NFAT stimulation, each will be added to separate wells for each time point. Anti-IL-2 ELISA tests will measure the amount of IL-2 production, a measure of T cell stimulation (and thus, antigen presentation) after the set time point of antigen uptake. By studying IL-2 levels from each T-cell strain at each time point, I hope to find when antigen uptake ceases, as indicated by a drop in IL-2 levels. By studying IL-2 levels for each T-cell strain in samples where both antigens were given, I hope to determine whether the DCs are capable of presenting both antigens.