UNIVERSITY OF SZEGED FACULTY OF MEDICINE

EDIT OLASZ M.D.

PRESENTATION OF SOLUBLE ANTIGENS AND HAPTENS BY MURINE BONE MARROW-DERIVED DENDRITIC CELLS – IMPLICATIONS FOR IMMUNOTHERAPY

Egyetemi doktori értekezés

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List of relevant publications

I. Olasz E.B., Linton J., Katz S.I. 2002. Soluble proteins and haptens on bone marrow-derived dendritic cells are presented to host CD4 T cells in an MHC-restricted manner. *Int Immunol* 14(5): 493-502.

II. Olasz E.B., Lang L., Seidel J., Green M.V., Eckelman W.C., Katz S.I. 2002. Fluorine-18 labeled mouse bone marrow-derived dendritic cells can be detected in vivo by high resolution projection imaging. *J Immunol Methods* 260(1-2): 137-48.

III. Saeki H., Wu M.T., Olasz E., Hwang S.T. 2000. A migratory population of skin-derived dendritic cells expresses CXCR5, responds to B lymphocyte chemoattractant in vitro, and co-localizes to B cell zones in lymph nodes in vivo. *Eur J Immunol* 30(10): 2808-14.

"Twenty years from now you will be more disappointed
by the things that you didn't do than by the ones you did do.
So throw off the bowlines. Sail away from the safe harbor.
Catch the trade winds in your sails.
Explore. Dream. Discover. "

Mark Twain

Dedication

I dedicate this dissertation to Aliz. Her beautiful eyes and happy smile kept me from giving up and helped to keep my feet on the ground.

Acknowledgements

I want to thank all my colleagues who provided a supportive environment at the Department of Dermatology at the University of Szeged, at the Dermatology Branch at the National Cancer Institute, Bethesda, Maryland, as well as at the Department of Dermatology at the Medical College of Wisconsin in Milwaukee, Wisconsin.

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Last, I want to thank John for all his love to me and Aliz.

Preface

The research of cutaneous immunology has a long tradition at the Department of Dermatology in Szeged (1). Main research studies focused on the pathomechanism of psoriasis with special emphasis on the involvement of interleukin-8 and its receptor in the diseased skin (2, 3). HHV8 DNA sequence was first reported in classic Kaposi sarcoma (4), angiolyphoid hyperplasia (5) and angiosarcoma of the face (6). Significant observations were made about the role of the innate immune system in psoriasis (7) and in cutaneous biology (8). The treatment of psoriasis (9) and vitiligo (10) by XeCl laser was first developed at the Department.

I joined the laboratory of Lajos Kemény as a medical student in 1992. This was the time when important findings regarding the role of the altered cytokine network in the accumulation of inflammatory cells in lesional psoriatic skin, and the cytokine involvement in epidermal hyperproliferation, led to new projects and ideas. New discoveries about the essential role of cytokines in the neutrophil and T cell accumulation in psoriatic skin paved the way for future biological therapeutics. The actions of important therapeutic compounds, such as corticosteroids, dithranol, cyclosporine, retinoids, vitamin D3 analogues and ultraviolet radiation were studied in collaboration with the laboratory of Drs. Thomas Ruzicka and Guenther Michel at the Deparment of Dermatology in Munich and later in Düsseldorf (11-13). Part of this collaboration led to my work on the demonstration of the interleukin-10 receptors on epidermal cells (14). Thanks to the excellent mentoring of Dr. Dobozy and Dr. Kemény I visited several other laboratories to study new methods and techniques involving both research and clinical fields. It was in the laboratory of Dr. Thomas Bieber at the Department of Dermatology in Munich that I developed an interest in the professional antigen presenting cells of the skin, the Langerhans cells (15).

One of the pioneers and a most influential researcher in the field of Langerhans cells has been Dr. Stephen Katz. In elegant experiments using bone marrow chimeras, he showed that Langerhans cells belong to the immune system by virtue of originating in bone marrow (16). Together with George Stingl, he made the first observations about the immunological functions of these cells (17, 18). He played a distinct role in the study of the patomechanism of contact sensitivity (19) and the induction of tolerance to contact sensitizers by UV light (20). By the late 80s and early 90s it became evident that dendritic cells are the professional antigen presenting cells of the body and they have tremendous potential for use in

immunotherapy protocols. However it was a challenge at that time to obtain these cells in large numbers for *in vitro* laboratory studies. In 1992 Inaba at al. published a novel method for culturing denditic cells from murine bone marrow (21), opening the doors for dendritic cell research. Upon my arrival at Steve's laboratory, my task was to set up the method for culturing mouse bone marrow-derived dendritic cells and studying their potential usefulness for *in vivo* immunotherapy protocols. This dissertation will discuss my work on characterizing bone marrow-derived dendritic cells, with special emphasis of their capability to present soluble protein antigens and haptens to CD4+ T cells *in vitro* and *in vivo*. Important observations regarding the antigen presenting potential of BMDC in comparison to Langerhans cells will be discussed. This work shows that soluble antigens and haptens are presented in an MHC-restricted manner. A novel method to detect and follow migration of in vitro cultured BMDC upon injection will be described.

Abbreviations

APC	**antigen presenting cell**
BCL	**B cell chemoattractant**
BM	**bone marrow**
BMDC	**bone marrow-derived cell**
CHS	**contact hypersensitivity**
CTL	**cytotoxic T lymphocyte**
CYT	**cytochrome c**
DC	**dendritic cell**
DCM	**dendritic cell medium**
DTH	**delayed type hypersensitivity**
EC	**epidermal cell**
GM-CSF	**granulocyt macrophage colony stimulating factor**
IL	**interleukin**
HEL	**hen egg lysosyme**
LC	**Langerhans cell**
LN	**lymph node**
mAb	**monoclonal antibody**
migDC	**migratory dendritic cell**
PAM	**proliferation assay medium**
PiPET	**Projection Imager/Positron Emission Tomograph**
PI	**propidium iodide**
SDF-1	**Stromal cell-derived factor 1**
SFB	**N-succinimidyl 4-[F-18]-fluorobenzoic acid**
SLC	**Secondary lympoid tissue chemokine**
sMS	**mouse serum**
TNCB	**trinitrochlorbenzene**

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1. Introduction

1.1. General introduction

The immune system is a complex network of cells, which protects the body from infectious agents and other foreign substances as well as internal pathological conditions such as tumor development. The most elaborate, dynamic, and effective defense strategies are carried out by cells that have evolved specialized abilities to recognize and eliminate potentially injurious substances. One of the central players of the immune system is the dendritic cell (DC), capable of initiating T-cell responses by taking up antigens and presenting them to T cells (22, 23). Although DCs comprise multiple subsets (24), all are unusually effective at antigen processing and presentation. DCs can take up a diverse array of antigens and present them to T cells as peptides bound to both MHC class I and II products. Relative to other antigen presenting cells, DCs are adept at stimulating naive T cells. DCs also control the quality of the T cell response, driving naive lymphocytes into distinct classes of effectors. These antigen-specific, adaptive responses are critical for resistance to infections and tumors. Conversely, DCs can also generate regulatory T cells that suppress activated T cells, a function of likely importance in autoimmunity and transplant rejection (25).

A novel approach to vaccination against cancer is to exploit DCs as "nature's adjuvants" and actively immunize cancer patients with a sample of their own DCs primed with tumor antigens (26). DC vaccination is, however, still at an early stage; important variables such as the source and method for propagating DCs, the route for immunization and antigen pulsing protocols – are currently under intensive investigation.

The principal mechanism by which the immune system eliminates tumors and viral infections is the cytotoxic T lymphoctyes (CTL) response. For tissue tropic viruses that do not infect APCs and for tumors of parenchymal cells, the APC must acquire and present antigens that are synthesized by the infected or tumor cell, a process that has been called "cross-presentation". Cross-presentation, when used in a broader sense, involves uptake of antigens from other cells. Previous studies elucidated some of the underlying mechanism of cross-presentation, particularly on MHC class I molecules. Particulate antigens are efficiently cross-presented after being internalized into APCs by phagocytosis. Consequently, DCs aquire cell-associated antigens for cross-presentation by phagocytosis of dying parenchymal cells and their debris.

The aim of this introduction is to give a brief overview about the role of dendritic cells in the initiation of MHC II-restricted immune responses, with special emphasis on bone marrow-derived dendritic cells. The introduction focuses on the mechanism of in vitro antigen uptake and in vitro and in vivo antigen presentation by dendritic cells and Langerhans cells to CD4 T cells. The mechanism of cross-presentation will be introduced. It will give a brief overview about DC migration, with special emphasis on the chemokine network. It provides information about the current status of DC immunotherapy.

1.2. Antigen-presenting cells and T-cell activation *in vitro*

DCs are potent stimulators of T cell responses and T-dependent antibody formation in tissue culture. Relatively few DCs and relatively low doses of antigen are required to elicit high levels of lymphocyte proliferation and differentiation. Initially, DCs had to be isolated directly from lymphoid tissues (or, in the case of humans, from blood), the scarcity of these cells imposed a serious limitation on DC research. Typically, DCs make up less than 1% of a given cell population, a figure that is somewhat misleading since the frequency of these cells is at least 100 times greater than that of T cells specific for any given antigen. Moreover, DCs are extensively ramified in regions of the lymph nodes through which T cells recirculate **(Fig. 1)**.

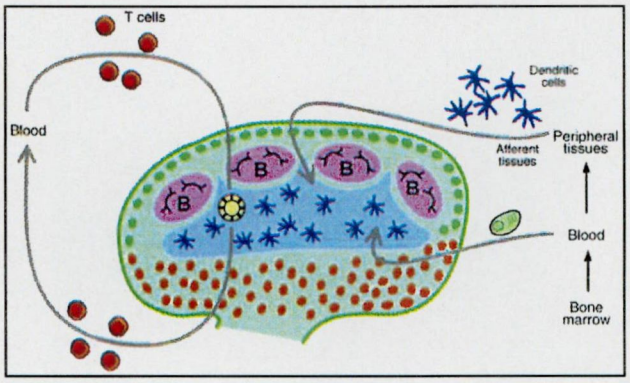


Figure 1. Lymphocyte and DC circulations. From Steinman et al. Exploiting dendritic cells for vaccine efficacy JCI 2002. 109. 1519-26.

First the general mechanism of Ag presentation will be discussed.

Most investigators now study DCs produced in much larger numbers from either $CD34^+$ proliferating progenitors or $CD14^+$ nonproliferating monocytic precursors. These DCs are charged or "pulsed" with antigens, which they efficiently process and display as MHC-peptide complexes. Antigen-pulsed DCs can be placed into culture with lymphocytes, whereupon T cells begin to proliferate and to produce lymphokines. The potency of DCs in stimulating T cells in vitro reflects both their specialized ability to capture and present antigens and the effects of other molecules, not present in MHC complexes, that enhance T cell binding and stimulation.

1.3. Antigen-presenting cells and T-cell activation *in vivo*

Vaccination with DCs leads to protective immunity against infections and tumors (27) and, in the case of certain self antigens, autoimmunity (28). DCs can be exposed to an antigen either in vivo, by introducing the antigen directly, or ex vivo, by pulsing the cells with antigens while they are in culture and administering them to genetically matched animals. After antigenic proteins are given to mice, DCs are found to be the main cells capturing antigen in an immunogenic form. When mice are challenged with microbes, DCs also are the principal cells producing the key protective cytokine IL-12. Ex vivo–activated DCs can prime recipient animals in an antigen-specific manner, allowing them to respond to an antigenic challenge within a week. These DCs migrate to the recipients' lymph nodes and lodge in the T cell areas, sites through which lymphocytes enter the lymph nodes via high endothelial venules **(Figure 1)**. This movement positions the DCs in a seemingly ideal niche to select antigen-specific T cells when the latter percolate through the node. Such selection can be observed directly *in situ*: Following activation in contact with DCs, the T cells leave the lymph node, freeing them to fight infections and tumors. Some also become memory T cells, a response whose mechanism remains to be unraveled.

1.4. DC maturation and its effect on antigen uptake

DCs in culture exist in two functionally and phenotypically distinct states, immature and mature. Immature cells are adept at endocytosis and express relatively low levels of surface MHC class I and II products and costimulatory molecules (e.g., CD86). Abundant MHC class II molecules are synthesized, but they are mainly sequestered intracellularly in late endocytic compartments. Antigens can be avidly taken up by immature DCs and targeted to MHC class II-positive lysosomes. However, they are not efficiently utilized for the formation of MHC II-peptide complexes, but are retained for use as immunogenic peptides days later. Thus, immature DCs in culture can take up antigen but do not present it efficiently to T cells. Most DCs in peripheral tissues in situ are of the immature phenotype, the prototype being Langerhans cells in the epidermis.

After detecting microbial products or proinflammatory cytokines, immature DCs transform into mature DCs, cells with a reduced capacity for antigen uptake but now with an exceptional capacity for T cell stimulation. This transition is accompanied by a dramatic

cytoplasmic reorganization highlighted by a redistribution of MHC class II from intracellular compartments to the plasma membrane. Class II molecules appear to exit from the lysosomes, then to reside transiently in nonlysosomal cytoplasmic structures (class II vesicles), and finally to accumulate on the cell surface. In tandem, surface costimulatory molecules (CD80, CD86, CD40), MHC class I, and T cell adhesion molecules (e.g., CD48 and CD58) are all upregulated. The maturing DCs also upregulate the capacity to generate functional peptide-MHC II complexes from newly internalized antigen or from antigen internalized prior to the maturation signal. The cells extend long "dendritic" processes that may increase opportunities for T cell capture and interaction. DCs also remodel their profile of chemokine receptors that facilitate homing to lymphoid organs.

Much of what is known concerning DC maturation has been learned from DC cultures, either cells differentiated with GM-CSF and IL-4 from non-growing human blood monocytes or from proliferating bone marrow-derived precursors

The current view has immature DCs encountering antigen in the periphery and carrying it to lymphoid organs, maturing en route. While valuable in general terms, this view is probably too simple for describing the DC system in vivo. For example, DCs migrating from the periphery may not always be the ones that present antigen in the lymph nodes. Rather, migrating DCs may transfer their captured antigens to other DCs for presentation. The transfer could occur either by phagocytosis of the antigen-loaded DCs (29) or by the release of antigen-bearing vesicles (exosomes) derived from a DC's lysosomal compartment (30). Another oversimplification is the idea that all DCs within a lymph node are mature. Most DCs within lymph node *in situ* may be able to form MHC-peptide complexes, but they are otherwise immature and may function to induce peripheral tolerance.

1.5. Langerhans cells as special members of the dendritic cell family

Langerhans cells (LC) are dendritic cells of the epidermis. In the epidermis and in other epithelia they reside mostly in a suprabasal position. Immunohistochemical en face views show that they form a regular network. Ultrastructurally, LC are characterized by a unique cytoplasmic organelle, the Birbeck granule.

As all cells of the immune system, LC stem from the bone marrow. This was originally found by Katz et al. and Frelinger et al. (16, 31) in the mouse, and by Perreault et al. and in humans (32). During ontogeny, LC precursors populate the epidermis and acquire

immunologically important molecules such as ATPase, CD45, MHC class II (33-35) and Langerin/CD207 as well as Birbeck granules in a step-wise fashion. LC has served as a preferred model to study dendritic cell biology. GM-CSF as the crucial survival and growth factor for dendritic cells was first established with LC (36). The concept of dendritic cell maturation was learnt from LC. However, there is increasing evidence that, in addition to the presence or absence of Birbeck granules, dendritic cells from the epidermis (i.e., LC) and dendritic cells from other tissues are not completely identical. Functional differences have been described (37, 38). For instance, LC-like dendritic cells take up less endocytic tracers such as fluorescein isothiocyanate dextran or peroxidase. The most striking difference is the failure of LC-like dendritic cells to induce naive B cells to differentiate into IgM-secreting cells, in response to CD40 triggering and interleukin-2, as opposed to interstitial type dendritic cells. Moreover, LC-like dendritic cells do not secrete IL-10 in response to CD40 ligation, suggesting that they may be better at inducing Th1 responses than their counterparts from the interstitium. It must be emphasized that these important functional specializations of LC have so far "only" been demonstrated with CD34+ stem cell-derived LC-like cells. Thorough side-by-side comparisons between LC isolated from the epidermis and dermal dendritic cells isolated from the dermis are still missing. At present, monocyte-derived dendritic cells (39) are the dendritic cells of choice for clinical applications, mainly because of their easy availability. Yet, LC may prove more efficient in the future. This dissertation will show differences in antigen presenting capacity between epidermal LC and BMDC.

1.6. Cross-presentation on dendritic cells

Early experiments examining the nature of MHC-restriction during T cell priming showed that minor histocompatibility antigens were capable of being transferred from those cells expressing these antigens to host APC (40). Priming involving this type of antigen transfer was termed "cross-priming" to differentiate it from direct T cell activation by the actual cells expressing the minor antigens. In recent times, such presentation has been given the term "cross-presentation" (41). According to this simple definition, cross-presentation can involve either class I- or class II-restricted antigens, although this term has often been associated solely with class I-restricted antigens. However, given that the same professional APC can present transferred antigen to both helper and killer T cells (42), a single term of cross-presentation is appropriate for both class I- and class II- restricted presentation of cell-

associated antigens. Cross-presentation of cellular antigens is involved in many different responses, including those to tumors, viruses, graft tissues and even self (43). *In vitro* studies have proposed several mechanisms by which antigen is transferred to APCs. In one model, dead (either necrotic or apoptotic) cells containing antigen are the main components of antigen transfer during cross priming (44). In this dissertation we will show that soluble and haptenated antigens presented on BMDC will not be taken up and represented by allogenic host APC *in vivo*.

1.7. Migration of DC

The migration and positioning of DCs are among the hallmarks of this lineage **(Fig. 1).** Antigen-bearing DCs must leave peripheral tissue via afferent lymphatic channels and eventually enter discrete regions of the secondary lymphoid organs, such as spleen and lymph nodes. At these sites, DCs complete their maturation, perhaps through mechanisms related to CD40 ligation, and potentially attract T and B cells through the release of chemokines (45). Chemokines and their corresponding seven trans-membrane-spanning, G-protein coupled receptors play important roles in the migration of DC and B cells to secondary lymphoid organs. Different chemokine receptors are valuable at different stages of the life history of DC. Among the chemokines, CCR2 seems important for DCs to translocate into the T cell rich regions of lymphoid tissues during contact allergy and infection with L. major (46); CCR5 may help recruit DCs to inflammatory sites (47, 48); CCR6 appears to be important for positioning DCs at epithelial surfaces (49); CCR7 may increase entry to lymphatics and migration to the T cell areas of lymph nodes (50). Inflammation and infection is not the only reason DCs migrate to lymphatics, DCs seem to continuously patrol through peripheral organs, lymph and lymphoid tissues in the steady state. This steady state migration provides DCs the opportunity to sample self-antigens and environmental proteins continuously for purposes of immune tolerance (51).

Although DC are known to interact with T cells and can migrate to T cell zones in secondary lymphoid organs (52), recent publications clearly demonstrate that DC have several direct effects on B cells (53). Different subpopulations of skin DC have been described, including epidermal Langerhans cell (LC) and dermal types. Recently significant differences were found between DC populations in skin. Allan et al. (54) showed that infection of murine epidermis by herpes simplex virus did not result in the priming of virus-

specific cytotoxic T lymphocytes by Langerhans cells. Rather, the priming response required a distinct CD8alpha+ dendritic cell subset. It has been found that only dermal-type DC have been found to stimulate naive B cells to synthesize antibodies (55). Recently, a $CD11c^+$ DC that was characterized by binding to a mannose receptor fusion protein (CRFc) has been demonstrated to accumulate in B cell zones of the LN, but whether they migrated there from the periphery or altered their surface phenotype within the LN itself is unclear (56). B cell chemoattractant (BLC) (57, 58) and its receptor, CXCR5 (58), have been shown to play an important role in B cell homing and lymphoid organogenesis (59). BLC is highly expressed in the B cell follicles of secondary lymphoid organs (57, 60) and mediates efficient migration of B cells *in vitro* (57, 58). To date, CXCR5 has only been shown to be expressed by B cells and a small subset of T cells (59).

Migratory DC (migDC) are skin-derived, activated DC that have migrated out of skin explants over the course of several days (61). MigDC express high levels of CCR7 and, when injected into the footpad of mice, they home to draining LN in an SLC-dependent fashion (62). In this study we show that migDC express 50-fold more CXCR5 mRNA than resting LC, respond to BLC *in vitro*, and are capable of migrating to B cell zones within LN *in vivo*. We propose that the expression of CXCR5 may be a mechanism by which DC migrate to B cell areas in secondary lymphoid organs.

1.8. Dendritic cells in immunotherapy - dose, frequency and route for dendritic cell delivery and dendritic cell migration

DCs are an attractive target for therapeutic manipulation of the immune system, to enhance insufficient immune responses in infectious diseases and cancer, or attenuate excessive immune responses in allergy and autoimmunity. The immunogenicity of antigens delivered on DCs has now been demonstrated in human studies (63). Indeed, single s.c. immunization of healthy volunteers with 2–4 × 10^6 antigen-loaded mature monocyte-derived DCs rapidly expanded CD8+ and CD4+ T cell immunity. A single boost several months later led to expansion of CTL with increased affinity against viral peptide, an observation never made with any other vaccination strategy so far (64). Vaccination with ex vivo generated DCs is not feasible for large-scale immunization, either in cancer or in infectious diseases, e.g., malaria. Thus, there is a need to develop strategies that can provide a robust protective/therapeutic immune response of optimal type with minimal amounts of vaccine and

limited boosting. Research in this area may benefit from the ability to (1) mobilize large numbers of DCs in vivo (DC-poietins) and (2) deliver mobilized DCs to antigens and activation molecules.

Targeting of antigen to DCs and the induction of their maturation will be important for any future *in situ* DC vaccination approach. Further work should also explore the induced migration of DCs and T cells into tumors to induce immunity against the total tumor antigen repertoire. The principal effectiveness of this pathway has been recently demonstrated by injection of chemokine-secreting DCs into tumors to prime T cells (65). The vaccination schedule and path of DC migration following vaccination have yet to be addressed and optimized in small immunogenicity trials. The optimal number of DCs will of course very much depend on the route and effectiveness of migration. Tumor antigen-specific T cells were unequivocally demonstrated following injection of DCs into the skin (either intradermal (66) or subcutaneous (67, 68), intranodal (69, 70) and intravenous (71) injection.

Migration of dendritic cells after intravenous, intradermal or subcutaneous injection has been assessed by using different tracing methods, such as membrane or cytosolic fluorescent dyes (72) (52) or cells labeled with radionuclides such as Cr-51 (73) or Indium-111 (74). In most of these studies the organs are removed at various times after injection of the label, preventing long term in vivo follow up of migrating cells. In the dissertation, we describe a new method to label DC with the positron emitting radionuclide F-18, in order to detect DC migration in vivo by a high-resolution small animal PET camera.

2. Aim of the studies

The general aim of the studies presented here is to investigate murine bone marrow-derived dendritic cells as model APCs for immunotherapy protocols. We used the soluble antigens hen egg-lysosyme and cytochrome c to test the capacity of BMDC to prime CD4+ T cells in vitro and vivo. We also tested these cells in contact hypersentivity experiments. Langerhans cells were compared to BMDC in regard to protein and hapten priming capability. The migration of BMDC upon subcutaneous injection was studied using flouorescent tracking and compared to migratory DC. The chemokine receptor CXCR5 on migratory DC was studied. We developed a novel positron emitting tomography method to follow BMDC migration in vivo.

2.1. To develop optimal BMDC culture and antigen pulsing method to maximize *in vitro* and *in vivo* protein-, and hapten-presenting capacity to CD4+ T cells

At the time of the study, there were few culturing methods for BMDC. Antigen pulsing conditions and antigen concentrations were not yet fully characterized. The challenge was to find optimal culturing and pulsing conditions in order to obtain highly mature BMDC capable to induce in vitro and in vivo antigen-specific proliferation of CD4+ T cells. Our goal was to functionally characterize bone marrow-derived cells (BMDC) to initiate $CD4^+$ T_h cell responses.

2.2. To investigate the differences between murine epidermal Langerhans cells and BMDC in phenotype and antigen-presenting capacity

Although Langerhans cells are considered as the dendritic cells of the epidermis, we hypothesized that functional differences exist between LC and BMDC. We used surface phenotyping with particular interest of costimulatory molecules and protein and hapten presentation assays to assess differences. What is the cell surface phenotype profile of LC? Are there differences between LC and BMDC in the capacity to present protein and haptenated antigens?

2.3. To assess whether murine BMDC efficiently present soluble proteins and haptenated antigens to CD4+ T cells *in vitro* and *in vivo*?

We used hen egg lysozyme and cytochrome c to assess the antigen presenting capability of BMDC. Our goal was to show that *in vitro* cultured BMDC present soluble

antigens and haptenated antigens *in vitro* and *vivo*. We used in vitro proliferation assays and in vivo contact hypersensivity and delayed type hypersensitivity experiments to answer these questions.

2.4. To assess whether there is cross-priming to CD4+ T cells *in vivo*

The mechanism of cross-presentation of exogenous antigens presented on MHC class I molecules has been shown to be transfer of peptide from apoptotic cells to DC. In contrast of our knowledge about the cross-presentation of antigens on MHC class I molecules, much less is known about presentation of antigens derived from other cells, on MHC class II molecules. Are protein and hapten-pulsed BMDC able to immunize allogenic host? Are proteins and haptens presented by injected BMDC are taken up by host APC and presented to the host in an immunogenic manner?

2.5. To assess the migratory properties of BMDC

Our goal was to show that injected BMDC migrate to the draining lymph nodes. We used intracellular tracking dyes to detect BMDC in draining LN upon injection to subcutaneous tissue.

2.6. To determine the differences in the migratory properties and chemokine receptor profile between BMDC and migratory DC

MigDC and BMDC express high levels of CCR7 and, when injected into the footpad of mice, they home to draining LN in an SLC-dependent fashion. We show that migratory DC are capable to migrate in the B cell zones. Our goal was to show that migDC express CXCR5 mRNA and respond to BCL in vitro.

2.7. To develop an imaging method to detect migration of BMDC *in vivo*

Our goal was to develop a method to label DC with the positron emitting radionuclide F-18, in order to detect DC migration in vivo by a high-resolution small PET camera. We describe our labeling method, the effects of labeling on the viability, phenotype and function of BMDC in vitro, and how F-18 labeled cells can be visualized and followed by a high-resolution animal scanner.

3. Materials and methods

3.1. Mice

Female BALB/c (I-A^d), C57BL/6 (I-A^b) and F_1 (CB6/F_1) mice aged 6–12 weeks old (Animal Production Area, National Institutes of Health, Frederick, MD) were used in all experiments, and were housed under pathogen-free conditions.

3.2. Bone marrow-derived cells

BMDC were generated using the method of Fields *et al.* (75) (**Fig 2**) with minor modifications. Briefly, BM was flushed from tibias and femurs of female BALB/c or C57BL/6 mice and depleted of red blood cells with ammonium chloride. Cells were plated at 1 x 10^6 cell/ml in T162 flasks in 50 ml DC medium [DCM: RPMI 1640 containing 5% FCS (Biofluids, Rockville, MD), 50 µM 2-mercaptoethanol (Sigma, St Louis, MO), 10 mM HEPES, pH 7.4, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 20 µg/ml gentamycin (all from Life Technologies, Chagrin Falls, OH), supplemented with 10 ng/ml granulocyte macrophage colony stimulating factor (GM-CSF) and 10 ng/ml IL-4 (PeproTech, Rocky Hill, NJ)]. On day 5, cultures were fed with an additional 25 ml of DCM. On day 7, non-adherent and loosely adherent cells were harvested by vigorous pipetting of cultures. Harvested cells were resuspended in RPMI + 5% FCS and 10 mM HEPES at 5 x 10^6 cells/ml. Then 10 ml aliquots of this cell suspension were layered onto 15 ml of 14.5% metrizamide (Sigma)/RPMI + 5% FCS and 10 mM HEPES solution in a 50 ml centrifuge tube and centrifuged at 1700 *g* for 10 min at 4°C. The resulting cell interphase was harvested, washed twice in RPMI + 5% FCS and 10 mM HEPES, and subcultured in DCM for an additional 1 day. On day 8, non-adherent cells were harvested and these cells are referred to as BMDC. The purity of BMDC as determined by FACS analysis was routinely 85–95%. Contaminating cells were mostly granulocytes and macrophages. No T or B cells were detected.

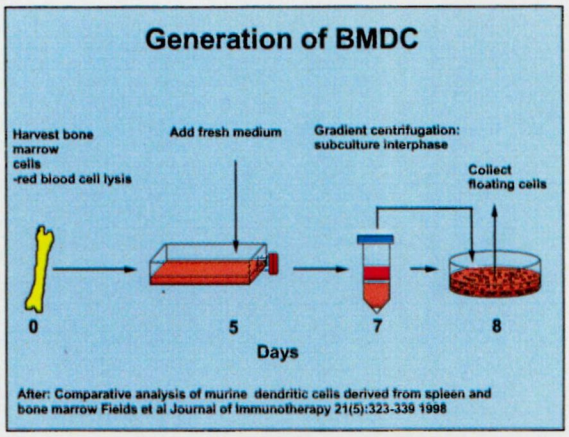


Figure 2. Culture protocol for generating BMDC

3.3. Epidermal cells (EC)

Single EC suspensions were obtained from ears of untreated BALB/c or C57BL/6 mice as previously described (76). Fresh LC were obtained by Lympholyte M (Cedarlane, Hornby, Ontario, Canada) gradient centrifugation as previously described (77) or further cultured (1.5 x 10^6 cells/ml) in RPMI 1640 containing 10% FCS (Biofluids) [or, in some experiments, with 1.5% syngeneic mouse serum (sMS)], 50 μM 2-mercaptoethanol (Sigma), 10 mM HEPES, pH 7.4, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids and 1 mM sodium pyruvate (cRPMI), which was supplemented with 10 ng/ml GM-CSF. After 48 h of culture (cLC), non-adherent EC were harvested by vigorous pipetting and LC were enriched by density gradient centrifugation (Lympholyte M). Interphase cells were washed extensively and stained with anti-I-A antibodies for FACS analysis to determine the percentage of LC (which was usually ~5–10% when freshly trypsinized cells were used and 25–30% when cLC were used.

3.4. Migratory DC

Murine migDC were isolated as described (61). Briefly, mouse ears were separated along the cartilage plane, scraped of excess fat and connective tissue, and placed epidermis-side up in cRPMI medium. After 3 - 4 days at 37 °C under 5 % CO_2, the skin was discarded and migratory cells were collected by centrifugation of the medium. Greater than 90 % of the collected cells expressed high levels of MHC class II by FACS and were > 95 % viable by propidium iodide exclusion.

3.5. Splenic B cells

Splenic B cells were isolated by preparing a suspension of spleen cells from BALB / c mice, labeling with FITC-conjugated anti-CD45R / B220, and positive selection using anti-FITC antibody-coated micromagnetic beads (Miltenyi Biotech. Berg, Gladbach, Germany) and passed through a magnetic isolation column. The resulting B cell population was > 95 % pure.

3.6. T cell hybridoma

The T cell hybridoma B9.1, specific for the immunodominant peptide HEL103–117 (78), was used to detect presentation of HEL by BMDC and LC, and was kindly provided by

Dr J. Kanellopoulos (Laboratoire de Biologie Moleculaire du Gene, INSERM U277, Institute Pasteur, Paris, France).

3.7. Flow cytometry

Expression of surface molecules was quantified by flow cytometry using the following antibodies: CD4 (H129.19), CD8a (53-6.7), CD11b (M1-70), CD11c (HL3), CD14 (rmC5-3), CD16/32 (2.4G2), CD40 (3/23), CD45R/B220 (RA3-6B2), CD54 (3E2), CD80 (16-10A1), CD86 (GL-1), Gr-1 (RB6-8C5) I-A^d (AMS-32.1), I-A^b (AF6-120.1) CD23 (B3B4, rat IgG2a), CD21 / 35 (7G6, rat IgG2b), CD45R / B220 (RA3-6B2), CD3e (145-2C11) and isotype controls, purchased as purified, FITC- or phycoerythrin-conjugated mAb from PharMingen (San Diego, CA). Anti-murine macrophage (F4/80) mAb were purchased from Serotec (Oxford, UK). Staining was performed according to standard techniques and cells were analyzed with a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, Mountain View, CA). Dead cells were excluded from all analysis by propidium iodide (100 nM; Sigma) staining.

3.8. *In vitro* protein antigen presentation assay

To prime T cells with proteins, either 100 µg of HEL or 100 µg of the non-cross reacting protein pigeon CYT (both Sigma) were emulsified in complete Freund's adjuvant (1:1) and injected into the left hind footpads of mice. After 7 days, popliteal lymph nodes were collected, pooled and $CD4^+$ T cells were purified with a mouse CD4 subset column kit (R & D Systems, Minneapolis, MN). In this and all of the other experiments, appropriate sMS was added to the media when T cells were purified for co-culture experiments in order to avoid T cell responses to FCS components. The resulting $CD4^+$ T cell preparations contained <0.2% I-A^+ cells. T cells were plated at 2 x 10^5 cells/well in 96-well flat bottom microplates (Costar, Corning Inc., Corning, NJ) in cRPMI 1640 containing 1.5% heat-inactivated sMS

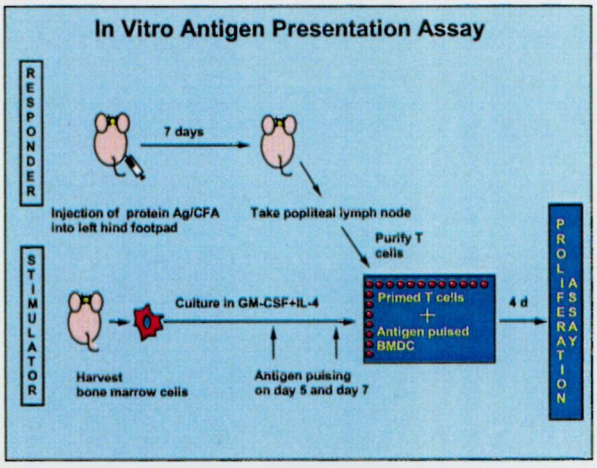


Figure 3. Protocol for in vitro proliferation assay

and supplemented with 1 μg/ml indomethacin (Sigma), referred to as proliferation assay medium (PAM). BMDC cultures were fed on day 5 with DCM containing various concentrations of HEL or CYT. DCs were purified by metrizamide gradient centrifugation on day 7. The interphase cells were washed extensively and replated at 5 x 10^5 cell/ml in DCM. On day 8, cells were washed, γ-irradiated with 3000 rad and added to T cells to achieve various stimulator:responder ratios. Freshly separated LC were enriched by gradient centrifugation and plated in 10 ml T flasks in complete RPMI containing various concentrations of HEL or CYT. After 48 h of culture, cells were harvested, washed 3 times in HBSS/1.5% sMS, γ-irradiated with 1500 rad and added to the T cells. On day 3 of co-culture, [^{3}H]thymidine (Amersham, Biosciences Corp., Piscataway, NJ) (1 μCi/well) was added and T cell proliferation was determined by the incorporation of [^{3}H]thymidine during the last 16 h of culture using a gas ionization counter (Packard, Meriden, CT). Results are presented as the mean (± SEM) of assays performed in triplicates. (**Fig 3.**)

3.9. Presentation of protein to antigen-specific T cell hybridoma cells *in vitro*

After incubation of BMDC or LC with HEL or CYT as described above, cells were added to 1 x 10^5 cells/well of B9.1 T cell hybridoma cells in 96-well flat-bottom microplates and cultured for 24 h at 37°C. One hundred microliters of culture supernatant was removed and assayed for IL-2 content using an ELISA assay (R & D Systems).

3.10. *In vivo* immunization with protein-pulsed BMDC

BMDC were pulsed with 1 mg/ml HEL or CYT as described above. Day 8 cells were washed 3 times with HBSS, and 3–5 x 10^5 antigen-pulsed and non-pulsed BMDC were injected into the left hind footpads of naive recipient mice. Seven days after immunization, draining popliteal lymph nodes were harvested and $CD4^+$ T cells were purified as described above. The resulting T cell population contained <0.2 % $MHC\ II^+$ cells. For assessment of intensity of the T cell

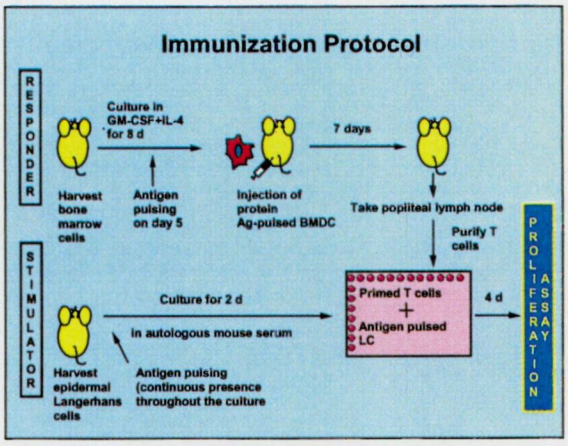


Figure 4. Immunization protocol

responses to the *in vivo* immunization, LC were pulsed as described above, γ-irradiated with 1500 rad, graded numbers of LC were added to 2 x 10^5 T cells/well and T cell proliferation was assessed as described above **(Fig. 4)**. In the cross-presentation experiments, allogenic HEL-pulsed or non-pulsed BMDC were prepared and injected as described above. In the proliferation assay LC syngeneic to the T cells were used as stimulators. The proliferation assays were performed in 1.5% sMS containing PAM. In the cross-presentation experiments using (BALB/c x C57BL/6) F_1 mice as hosts, both BALB/c and C57BL/6-derived LC were used as stimulators, and 1.5% F_1 sMS was used in the stimulator assays.

3.11. Delayed-type hypersensitivity (DTH) assay

To sensitize for protein antigens, mice were injected s.c. at two sites in the lower abdomen either with 2–5 x 10^5 protein-pulsed BMDC in 100 µl HBSS solution or, as a positive control, with 100 µl HEL/complete Freund's adjuvant. Non-pulsed BMDC and naive, non-sensitized animals served as negative controls. Challenge and measurement of ear thickness was performed as previously described (79). Each experimental group contained 10 mice.

3.12. Hapten modification of cells

Day 8 BMDC and cLC were TNP conjugated as previously described (77). Briefly BMDC were washed three times with HBSS, incubated with 1 mM TNBS (Sigma, St. Luis, MO) in HBSS for 10 minutes at 37 °C. After incubation cells were washed 3 times with HBSS+5% FCS. Trypan blue exclusion revealed 90% cell viability.

3.13. Sensitization and elicitation of contact hypersensitivity

Graded numbers (10^4–10^6 cells/mouse) of TNP-modified and unmodified BMDC and LC were injected in 100 µl HBSS/5% FCS s.c. into the dorsal trunk skin of BALB/c, C57/BL6 or F_1 mice. As positive controls, mice were sensitized by epicutaneous application of 100 µl 3% trinitrochlorbenzene (TNCB; Polysciences, Warrington, PA) in acetone:olive oil (4:1) to the dry-shaved abdomen. As negative controls mice were painted with the vehicle alone on the abdomen. Challenge and measurement of ear thickness was performed as previously described (77). Experimental groups consisted of five mice each. One-way ANOVA was used to compare experimental and control groups.

3.14. Presentation of TNBS by BMDC to TNCB-sensitized $CD4^+$ cells *in vitro*

Mice were painted with 100 µl of 3% TNCB as previously described (77). Brachial, axillary and inguinal lymph nodes were harvested 6 days later, purified for $CD4^+$ cells and plated in 96-well plates at 2 x 10^5 cells in 100 µl/well. BMDC were conjugated with TNBS as previously described, washed extensively and added to wells in various numbers. Proliferation assays were performed as described above **(Fig. 5)**.

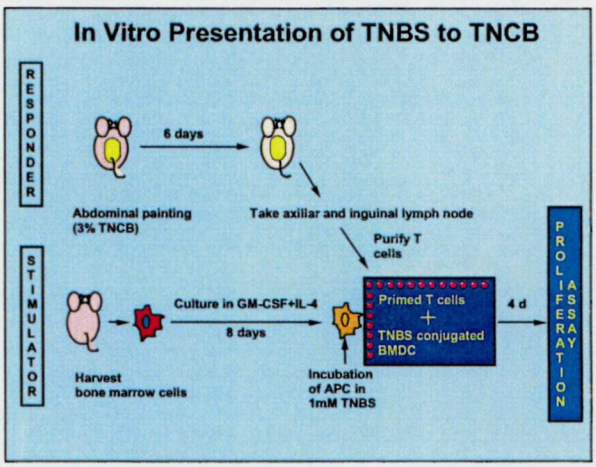


Figure 5. Protocol for in vitro presentation of TNBS to TNCB primed T cells

3.15. Assessment of secondary responses of lymph node T cells sensitized by TNBS-conjugated BMDC

BALB/c-derived BMDC were conjugated with TNBS, washed extensively and resuspended in HBSS. TNBS-conjugated or non-conjugated BALB/c-derived BMDC (3–5 x 10^5) were injected in 50 µl into the hind footpads of BALB/c and C57BL/6 mice. Draining popliteal lymph nodes were harvested 6 days later and purified for $CD4^+$ cells. As stimulator cells, we used BALB/c- and C57BL/6-derived LC, which were cultured in 1.5% sMS-containing medium for 2 days and conjugated with TNBS. MHC-matched stimulators and responder cells were co-cultured in the appropriate sMS-containing PAM and proliferation assays were performed

3.16. In vitro assessment of *in vivo* cross priming of haptens and soluble proteins presented by injected BMDC

To assess whether haptenated or protein-pulsed BMDC are capable to prime allogenic host we injected Balb/c-derived

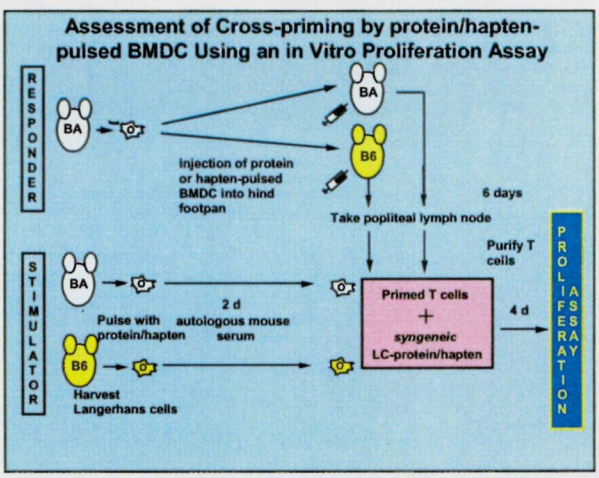


Figure 6. Protocol for in vitro assessment of cross-priming by protein/hapten-pulsed BMDC

haptenated or protein-pulsed BMDC onto Balb/c and C57BL/6 mice. 6 days later draining popliteal LN were taken and purified T cells were stimulated with hapten-conjugated or protein-pulsed syngeneic LC (**Fig. 6**).

3.17. Quantitative PCR

Commercial kits for RNA extraction (RNeasy, Qiagen, Valencia, CA) and reverse transcription (Superscript Preamplification System, Gibco-BRL, Gaithersburg, MD) were used prior to amplification of specific cDNA using the following primer pairs selected from Genbank sequences listed in 5' to 3' direction: CCR7 (GGACACGCTGAGATGCTCACT and CCATCTGGGCCACTTGGAT), CXCR5 (GACTCCTTACCACAGTGCACCTT and GGAAACGGGAGGTGAACCA). Murine glyceraldehyde 3-phosphate dehydrogenase (CGTGTTCCTACCCCCAATGT and TGTCATCATACTTGGCAGGTTTCT) was used as an internal control for loading. Real-time quantitative RT-PCR (Perkin-Elmer ABI7700) was performed with duplicate samples using SybrGreen dye for detection of double-stranded DNA. All primer pairs were designed to give products between 65 and 75 bp in length, gave rise to single band products, and had similar efficiencies of exponential amplification. Cycle threshold numbers (Ct) were derived from the exponential phase of PCR amplification. Fold differences in expression of gene x in cell populations y and z were derived by 2^k, where $k = (Ct_x - Ct_{G3PDH})_{\text{population y}} - (Ct_x - Ct_{G3PDH})_{\text{population z}}$.

3.18. *In vivo* migration of skin derived murine DC and BMDC

BMDC and MigDC (300,000 cells) were either labeled with PKH26 (Sigma, St. Louis, MO) according to manufacturer's instructions or left unlabeled and injected into the left hind footpad of mice. The left popliteal LN was removed 24, 48, or 72 h later, sectioned (6 μm), air-dried without fixation, and stained with FITC-conjugated antiCD45R / B220 to identify B cell areas. T cell areas were stained directly with FITC-conjugated anti-CD3e. When unlabeled DC were injected, LN sections were stained with biotin-conjugated anti-CD11c followed by streptavidin-Cy3 to identify DC. Isotype control staining showed negligible nonspecific staining. Day 8 mature live or heat-killed (50 min at 56 °C) BMDC were labeled with the fluorescent dye PKH67 (Sigma, St. Louis, MO) and injected ($3–5\times10^5$ cells in 50 μl PBS) s.c. into left hind footpad of mice. The left popliteal LN was removed 48

h later, sectioned (6 μm), air-dried without fixation and observed under the fluorescence microscope.

3.19. Preparation of N-succinimidyl 4-[F-18]-fluorobenzoate (SFB)

The F-18 labeled active ester was prepared in a three-step method. First, the trimethylanilinium triflate leaving group was displaced in the presence of Kryptofix (K-222) (Aldrich, Milwaukee, WI) and potassium carbonate (J. T. Baker, Phillipsburg, NJ) at 100 °C for 10 min with [F-18] fluoride. Next the pentamethylbenzyl ester was hydrolyzed to form 4-[F-18]fluorobenzoic acid with trifluoroacetic acid at room temperature for 2 min. Finally, the acid was converted to the F-18 labeled active ester, N-succinimidyl 4-fluorobenzoate **(Fig. 7)**, with *N,N'*-disuccinimidyl carbonate at 90 °C in 5 min. The final product was purified with reversed-phased HPLC.

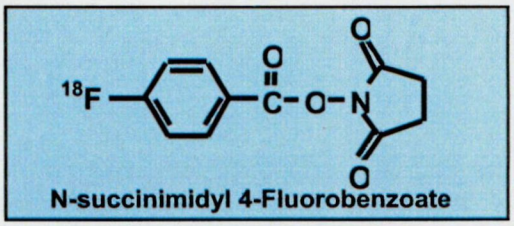


Figure 7. Chemical structure of SFB

3.20. Radiolabeling

1–3×10^7 BMDC in 1 ml HBSS (pH=8) were labeled with 5–10 mCi of [F-18]SFB at room temperature for 10 min. The cells were washed extensively with HBSS before labeling to remove FCS from the medium. Following incubation, the cells were washed three times with HBSS +5% FCS. Once the labeling and washing procedures had been completed, the labeling efficiency was measured with a gamma counter (Amersham Radioisotope Calibrator ARC-120). All subsequent F-18 counts described in the following sections were corrected for physical decay. The specific activity of the labeled cells was between 1 and 2 mCi per 1–3×10^7 BMDC. At a specific activity of F-18 at EOB of 200 mCi/μmol, this would result in >10^7 F-18 per cell.

3.21. Measurement of BMDC viability and radiolabel stability

Viability of F-18-labeled BMDC was established by trypan blue (0.4%) (GibcoBRL) exclusion in a Burker hematocytometer after 24 h in culture. Viability is expressed as percentage of viable cells among the total number of cells. The retention of radionuclide with BMDC was assessed by incubating F-18-labeled BMDC in DC medium at 4 °C or at 37 °C.

Cells were incubated for 4 h. At 1 h intervals, equal aliquots were centrifuged for 5 min at 1200 rpm at 4 °C and cell and supernatant were assessed for radioactivity using a Packard 5600 gamma counter (Packard Instrument, Meriden, CT).

3.22. Mixed lymphocyte reaction

BALB/c-derived F-18-labeled and sham-treated BMDC were washed extensively, γ-irradiated with 3000 rad, and added in graded numbers to allogeneic C57BL/6-derived naive T cells, which were harvested from peripheral lymph nodes and purified for $CD4^+$ T cells with CD4 columns (R&D Systems, Minneapolis, MN). Proliferation assay was perforemed as described above. T cell proliferation was determined by the incorporation of ^{3}H-thymidine during the last 16 h of culture using a gas ionization counter (Packard, Meriden, CN). Results are presented as the mean±SEM of assays performed in triplicate.

3.23. Chemotaxis assay

For the CXCR5 studies, murine migDC were used in chemotaxis assays as described (62) using 8μm (for migDC) and 5μm (for BMDC) pore size filters. Cells (25 μl containing approximately 20,000 cells) were placed on top of the filters in duplicate. After 3 h at 37 °C, migrated cells that had fallen to the bottom of the plate were photographed using a 4 × objective. Four random views from two wells were counted using Image Pro Plus (Media Cybernetics). For the F-18 studies, BMDC were labeled with the intracellular fluorescent dye, calcein-AM (Molecular Probes, Portland, OR), at 1 μM for 30 min at 37 °C, washed extensively with HBSS and subsequently labeled with F-18, as described above, or with PBS (sham treated). A total of 25×10^4 cells in 25 μl of RPMI, 0.5% BSA, and 10 mM HEPES medium were loaded on the top of the filter (ChemoTx 5 μm, Neuroprobe, Gaithersburg, MD). Murine secondary lymphoid tissue chemokine (SLC) (R&D Systems) was placed in different concentrations in the bottom well to attract BMDC. After 4 h at 37 °C in a humidified incubator, the cells accumulating at the bottom of the chamber were enumerated in four randomly chosen 10× views and averaged. Results are expressed as the mean±SEM for duplicate wells. Recombinant chemokines were purchased from R&D Systems, Minneapolis, MN (murine BLC) or Peprotech, Rocky Hill, NJ (human SDF-1 and murine SLC).

3.24. Positron imaging

Based on the results of tissue assays, we elected to perform the initial "proof-of-principal" imaging experiments on animals sacrificed 4 h post-injection rather than on living animals. Tissue assays indicated that a very large fraction of the injected activity accumulated in the bladder, that much of the material remained in the footpad and that the smallest amount of activity was distributed over several different organs. In order to maximize the probability of detecting LN activity under these conditions, the bladder was removed from each animal before imaging to eliminate this high activity source from in, or near, the imaging field-of-view. In addition, these experiments were performed with the PiPET (Projection Imager/Positron Emission Tomograph) scanner configured for planar projection imaging **(Fig. 8)**. Although this 2-mm resolution device is capable of high-resolution rotational positron emission tomography, planar projection imaging is less susceptible to statistical uncertainties since the depth dimension is collapsed onto a two-dimensional plane.

BALB/c mice, injected with 1×10^6 (~400 μCi) F-18-labeled BMDC, were prepared as described above and imaged for 10 min with PiPET such that the lower half of each animal, including the legs, was within the 55 mm×45 mm field-of-view. The animal was imaged again and these two views combined to create a decay corrected whole-body image of the entire animal. All images were analyzed using the NIH Image software package to obtain estimates of LN activity, footpad activity and background activity. The ratio of net LN/net footpad activity was then determined for each study for comparison with the results from tissue assays. The images were also inspected qualitatively to assess the apparent biodistribution of the injected BMDCs at 4 h.

Figure 8. Small animal PiPet

3.25. Statistical analysis

Experiments were performed at least twice. Mean differences were considered significant when $p<0.05$ (Student's *t*-test and one way ANOVA).

CHAPTER I.

4. Results I.

4.1. Culturing conditions for obtaining mature BMDC

We followed Fields at al. (75) protocol, which omits depletion of lineage positive cells at the initiation of the culture and utilizes a gradient centrifugation step on day 7 to remove contaminating non-DCs. Day 8 BMDC cultured with GM-CSF/IL-4 supplemented media expressed high levels of MHC class II molecules, high levels of CD86, CD80 and CD54 co-stimulatory molecules, and were positive for CD11c and CD11b, and negative for CD14 **(Fig. 9)**. Few cells expressed F4/80 molecules.

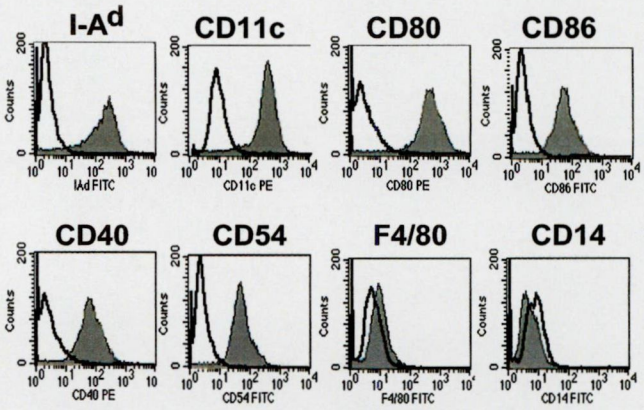


Figure 9. Cell surface phenotype of BMDC harvested on day 8 cultured with GM-CSF and IL-4. Cells were stained as described in Methods. Cells express high levels of MHC class II molecules, moderate to high levels of CD86, CD80 and CD54 co-stimulatory molecules, were positive for CD11c and CD11b, and negative for CD14. Few cells expressed F4/80 molecules. Shaded areas, mAb of interest; unshaded areas, isotype control mAb. Representative data from one of five experiments.

Figure 10 shows phase microscopic picture of BMDC clusters on day 7 and mature veiled BMDC on day 8. These findings suggest that 8-day BMDC, which we used in all of our experiments, exhibited a highly mature phenotype. Immature BMDC formed loosely adherent aggregates on day 6 of culture, while on day 8, BMDC appeared as single, veiled cells.

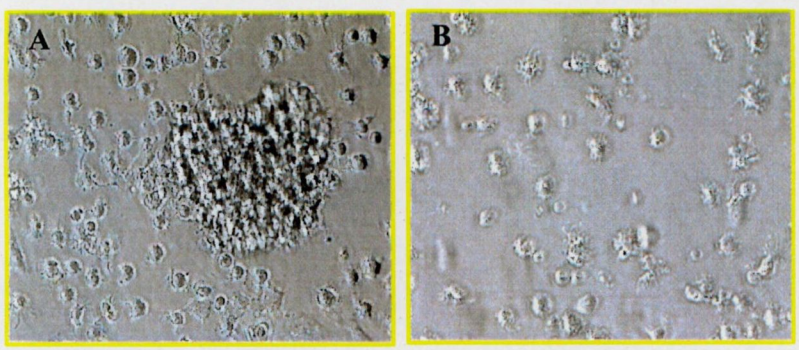


Figure 10. Phase contrast microscopy pictures of BMDC. Picture A) shows loosely adherent aggregates of BMDC on 6 days of culture, B) shows non-adherent veiled mature BMDC on day 8 of

4.2. BMDC process and present soluble protein antigens in association with MHC II molecules

To assess the *in vitro* antigen-presenting capacity of BMDC, the cells were either left unpulsed or pulsed with different concentrations (0.1–3 mg/ml) of HEL or CYT and graded numbers of these cells were added to fixed numbers of HEL- or CYT-primed T cells. BMDC pulsed with 0.1 mg/ml of protein induced little (and in some experiments, no) proliferation of primed T cells. Only BMDC pulsed with high concentrations ($0.5 < 1 \leq 3$ mg/ml) of protein were able to regularly induce significant antigen-specific T cell proliferation **(Fig. 11)**. In subsequent experiments, we used 1 mg/ml protein for pulsing because this concentration consistently induced significant T cell proliferation.

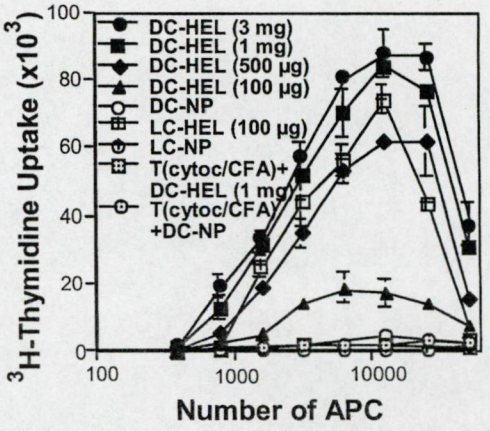


Figure 11. High concentration of protein is required to pulse BMDC in order to achieve significant antigen-specific proliferative response of primed T cells *in vitro*. BMDC were pulsed on day 5 of culture with various concentrations (100 µg, 500 µg, 1 mg and 3 mg) of HEL (BMDC-HEL, closed symbols) or left non-pulsed (BMDC-NP, open symbol). Langerhans cells were pulsed with 100 µg HEL (LC-HEL) or left unpulsed (crossed symbols). On day 7, BMDC were purified by gradient centrifugation and subcultured overnight. Day 8 BMDC and 2-day cultured LC were washed extensively to remove excess antigen, irradiated and co-cultured with 2×10^5 $CD4^+$ T cells purified from draining popliteal lymph nodes 6 days after mice were injected with 100 µg HEL/complete Freund's adjuvant or with 100 µg CYT/complete Freund's adjuvant (hatched symbols) as negative control. The LC number was normalized by the percentage of class II^+ cells by flow cytometry in this and all other experiments. Proliferation assays were performed in 1.5% sMS-containing medium in this and all other experiments. When cultured alone, T cells [non-stimulated or staphylococcal enterotoxin B (10 µg/ml) stimulated] as well as irradiated stimulators did not proliferate. In this and all other experiments cell proliferation was measured on day 4 by counting 16 h incorporation of [^{3}H]thymidine (mean ± SE in triplicate wells). Representative data from one of four similar experiments are shown.

4.3. Cultured LC process and present soluble protein antigens and induce enhanced secondary T_h cell responses compared to BMDC

When the antigen-presenting capacity of BMDC and LC were compared using different concentrations (0.1–3 mg/ml) of protein antigen, LC induced highly specific T cell proliferation at lower protein-pulsing concentrations (0.1 mg/ml) **(Fig. 11)**. If, however, higher concentrations were used, BMDC were as potent stimulators as LC, suggesting either

that LC might capture or process antigen in a more efficient manner, or that LC express co-stimulatory molecules that function more effectively than those expressed by BMDC. Surface phenotype by FACS analysis showed that cLC express CD40 costimulatory molecules seven times higher intensity as compared to BMDC and αCD40 Ab matured BMDC **(Fig. 12)**. Although this comparison may not provide an important distinction between the two cell types because of the differences in isolation techniques and in culture conditions, the results indicate that the two cell types may differ in their ability to present antigen.

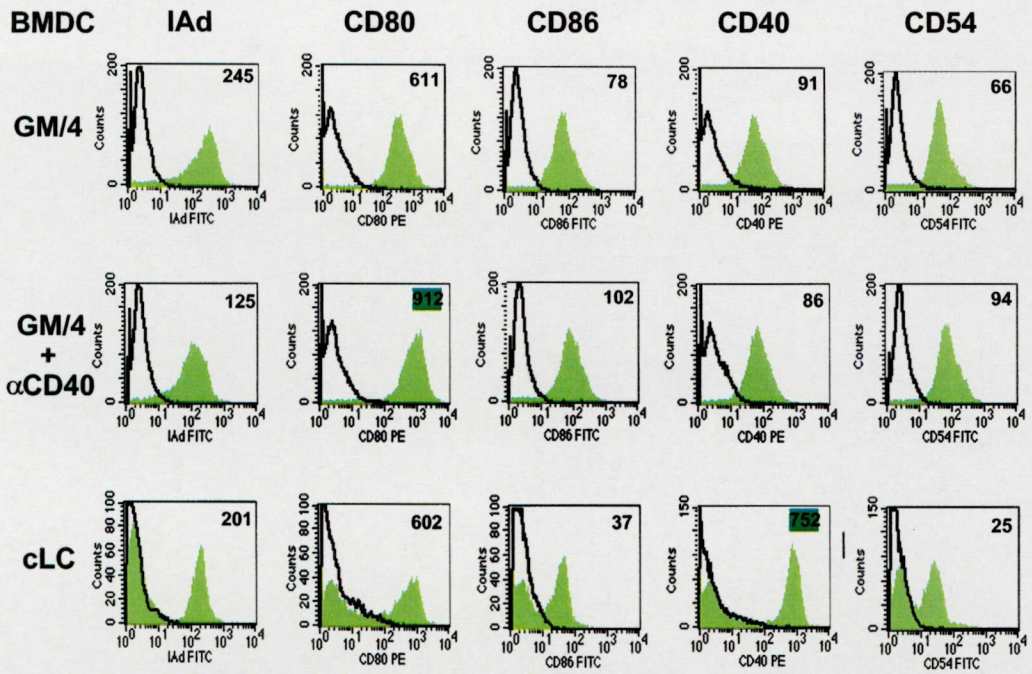


Figure 12. Comparative cell surface phenotype of 1) 8d BMDC harvested cultured with GM-CSF and IL-4, 2) BMDC harvested cultured with GM-CSF and IL-4 for 7 days and stimulatory αCD40 Ab for an additional 2d, 3) LC cultured for 3 days with GM-CSF and IL-4. Maturation with aCD40 upregulates CD80 expression on BMDC. cLC express similar levels of MHCII, CD80 molecules, but much higher level of CD40 compared to BMDC. Shaded areas, mAb of interest; unshaded areas, isotype control mAb. Representative data from one of five experiments.

4.4. Presentation of HEL to HEL-specific T cell hybridoma cells

To evaluate the capacity of BMDC to present HEL to the HEL-specific T cell hybridoma B9.1, BMDC were pulsed with different concentrations of HEL or CYT (as control antigen) and incubated with the hybridoma cells for 24 h. BMDC pulsed with as little as 10 μg/ml HEL were able to induce specific T cell proliferation as determined by significant

IL-2 production (**Fig. 13**). This result probably reflects the fact that T cell hybridomas require less antigen–MHC class II complexes on the APC surface and little or no co-stimulation in order to secrete IL-2.

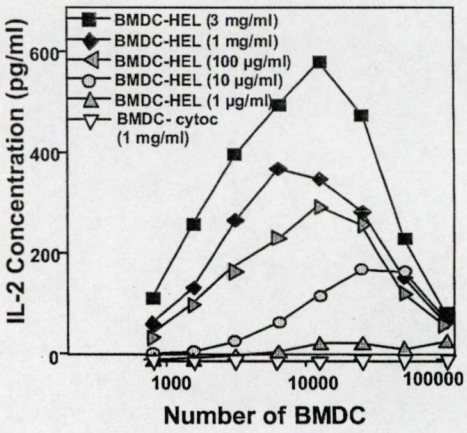


Fig. 13. IL-2 production of the HEL-specific T cell hybridoma B9.1 in response to antigen-pulsed BMDC. BMDC were pulsed with various concentrations (1 µg, 10 µg, 100 µg, 1 mg and 3 mg) of HEL (BMDC-HEL) or 3 mg CYT (BMDC-cyto), harvested on day 8 and washed extensively to remove excess antigen. Irradiated cells were added to 1 x 10^5 cells/well of B9.1 T cell hybridoma in 96-well flat-bottom microplates and cultured for 24 h at 37°C. Aliquots of 100 µl of culture supernatant were removed and assayed for IL-2 content as a measure of antigen-specific T cell stimulation, using an IL-2 ELISA assay. T cells alone and irradiated BMDC alone did not produce measurable amounts of IL-2. One experiment representative of three is shown.

4.5. Soluble antigen-pulsed BMDC prime lymph node T cells *in vivo* in an MHC-restricted manner

After pulsing with 1 mg/ml of HEL or CYT, as described above, 3–5 x 10^5 BALB/c-derived BMDC were injected into the left hind footpad of BALB/c and C57BL/6 (I-A^d and I-A^b) mice. Draining popliteal lymph nodes were harvested, pooled, and $CD4^+$ T cells were purified and assessed for secondary responses to antigen-pulsed APC. As APC, HEL- or CYT-pulsed cLC were used and added to the syngeneic purified lymph node T cells. Antigen-pulsed LC specifically stimulated those lymph node T cells primed with antigen-pulsed syngeneic BMDC, but not those primed with allogeneic BMDC **(Fig.14)**. This finding indicates that priming occurs in an MHC-restricted manner and, more importantly, shows that there is no cross-presentation by host APC. It excludes the possibility that there is representation of antigens by autologous APC.

To further investigate the phenomenon of cross-presentation on MHC II molecules, we injected 3–5 x 10^5 HEL-pulsed and non-pulsed BALB/c- and C57BL/6-derived BMDC into the footpad of F_1 (BALB/c x C57BL/6) mice. The draining lymph nodes were harvested

7 days later and $CD4^+$ T cells were purified and assessed for secondary T cell responses as described above. Stimulator HEL-pulsed and non-pulsed LC from BALB/c and C57BL/6 mice were cultured for 2 days in sMS-containing medium, and the proliferation assay was performed in each condition in PAM supplemented with 1.5% serum obtained from F_1 mice. BALB/c-derived HEL-pulsed stimulator LC could not induce secondary proliferation of T cells primed with HEL-pulsed C57BL/6 BMDC. In parallel, C57BL/6-derived HEL-pulsed LC did not induce secondary proliferation of T cells harvested from F_1 mice primed with BALB/c-derived HEL-pulsed DC (**Fig. 15**). These findings indicate that peptides presented in association with one of the MHC II type molecules could not induce clonal T cell expansion of T cells with TCR specific for the other MHC class II molecule.

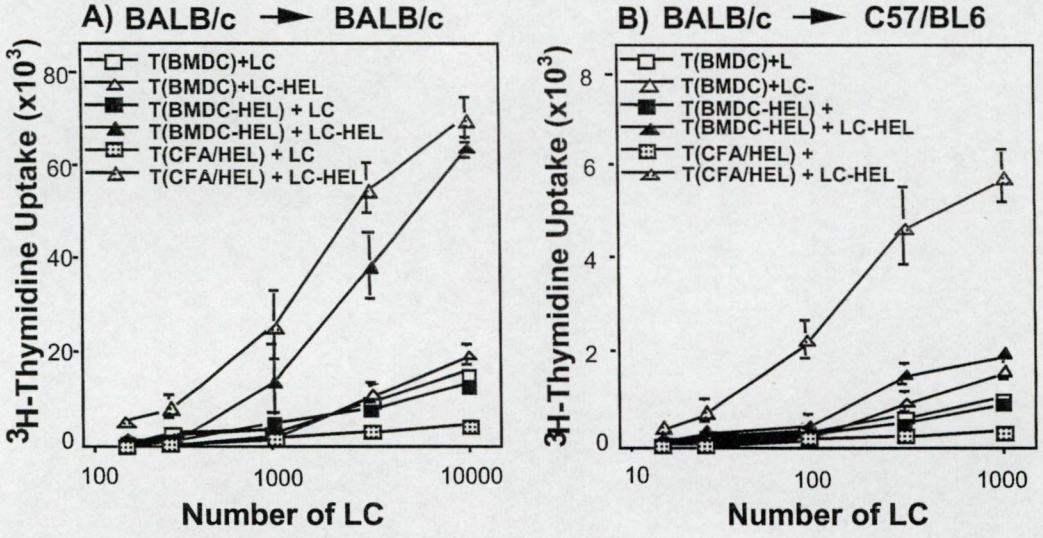


Figure 14. Priming for soluble protein antigens occurs in an MHC-restricted manner. Primary sensitization of BALB/c (A) and C57BL/6 (B) mice was attempted by injection of 3–5 x 10^5 BALB/c BMDC that had been left unpulsed (open symbols) or were pulsed (closed symbols) with 1 mg/ml HEL, or by injection with 100 μg HEL in complete Freund's adjuvant (hatched symbols). Stimulator cells in the secondary response were unpulsed (squares) or pulsed (triangles) BALB/c- or C57BL/6-derived cultured LC, which were irradiated, washed extensively and plated in 96-well flat-bottom tissue culture plates in graded numbers with 2 x 10^5 purified BALB/c or C57BL/6 $CD4^+$ lymph node T cells. T cells primed with s.c. injection of syngeneic HEL-pulsed BMDC proliferated in response to syngeneic HEL-pulsed LC, in contrast to T cells primed by allogeneic HEL-pulsed BMDC that did not proliferate. When cultured alone, T cells [non-stimulated or staphylococcal enterotoxin B (10 μg/ml) stimulated] as well as irradiated stimulators did not proliferate. One experiment representative of three is shown.

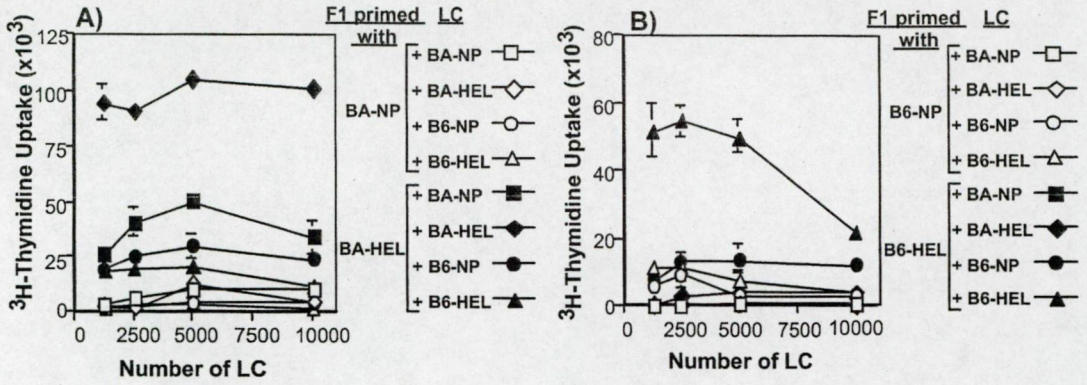
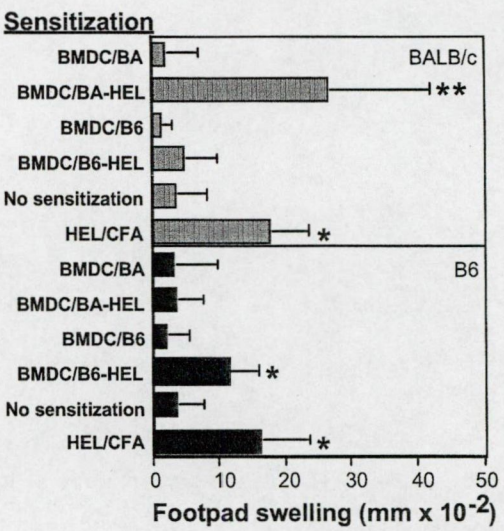


Figure 15. BMDC prime F_1 T cells in an MHC II-restricted manner. (A) Primary sensitization of F_1 (BALB/c x C57BL/6) mice was attempted by injection of 3–5 x 10^5 BALB/c BMDC that had been left unpulsed (open symbols) or were pulsed (closed symbols) with 1 mg/ml HEL. Stimulator cells in the secondary response were unpulsed (squares) or pulsed (diamonds) BALB/c-derived cultured LC and unpulsed (circles) and pulsed (triangles) C57BL/6-derived cultured LC, which were irradiated, washed extensively and plated in 96-well flat-bottom tissue culture plates in graded numbers with 2 x 10^5 purified F_1 $CD4^+$ lymph node T cells. (B) Primary sensitization of F_1 mice was attempted by injection of 3–5 x 10^5 C57BL/6 BMDC that had been left unpulsed (open symbols) or were pulsed (closed symbols) with 1 mg/ml HEL. Stimulator cells in the secondary response were unpulsed (squares) or pulsed (diamonds) BALB/c-derived cultured LC and unpulsed (circles) or pulsed (triangles) C57BL/6-derived LC, which were irradiated, washed extensively and plated as described in (A). HEL-pulsed LC induced significant secondary proliferation of those F_1 T cells primed with syngeneic HEL-pulsed BMDC. In contrast, T cells primed by HEL-pulsed BMDC allogeneic for the stimulators did not proliferate. When cultured alone, T cells [non-stimulated or staphylococcal enterotoxin B (10 μg/ml) stimulated] as well as irradiated stimulators did not proliferate. One experiment representative of three is shown.

4.6. BMDC induce DTH in an MHC-restricted manner



To further evaluate the immunogenic potential of protein-pulsed BMDC *in vivo* we assessed the mice for expression of DTH. A single s.c. injection of HEL-pulsed BMDC (4–10 x 10^5 cells/mouse), but not unpulsed BMDC, led to successful immunization of both BALB/c and C57BL/6 mice. Next we injected HEL-pulsed BALB/c- and C57BL/6-derived BMDC into syngeneic or allogeneic hosts to assess whether allogenic BMDC were able to sensitize mice

Figure 16. DTH response of mice immunized with syngeneic or allogeneic HEL-pulsed BMDC. BALB/c- and C57BL/6-derived BMDC were pulsed with 1 mg/ml HEL (BMDC/BA-HEL and BMDC/B6-HEL) or left unpulsed (BMDC/BA and BMDC/B6). Mice were injected s.c. at two sites in the lower abdominal skin either with 2–5 x 10^5 cells in 100 µl HBSS solution or with 100 µl HEL/complete Freund's adjuvant (2 mg HEL/ml) as a positive control. Naive, non-sensitized animals served as negative controls. Six days later mice were challenged by injecting 25 µl of HEL in PBS (2 mg/ml) s.c. into the left footpad, whereas as a control 25 µl of PBS was injected into the right footpad. After 24 h, footpad thickness was measured and the footpad-swelling response was calculated as the thickness of the left footpad minus the swelling of the right footpad. Only syngeneic HEL-pulsed BMDC were able to sensitize for a DTH response; neither non-pulsed nor HEL-pulsed allogeneic BMDC could prime for HEL. Data are representative of three independent experiments, showing the mean ± SEM (n = 10) of the footpad swelling response. *P < 0.05 and **P < 0.01 assessed by one-way ANOVA.

for HEL. Only when the injected cells were syngeneic to the recipient was a significant foot-swelling response detected. In contrast, if we injected allogeneic HEL-pulsed BMDC, no significant responses were detected (Fig. 15). This *in vivo* cross-priming experiment demonstrated that soluble antigen-pulsed BMDC cannot immunize allogeneic hosts for protein antigen-specific responses.

4.7. Hapten-modified BMDC and hapten-modified LC induce comparable amounts of proliferation of antigen-specific T cells

We next determined whether BMDC cultured in this way were able to present haptenated antigens *in vitro*. We sensitized the mice with 3% TNCB on the abdomen as described above, and harvested the brachial, axillary and inguinal lymph nodes 7 days later. We purified the lymph node cells for $CD4^+$ cells as described above and used them as responders in standard proliferation assays. As stimulators, we used BMDC conjugated or non-conjugated with TNBS. TNBS-conjugated BMDC were able to induce secondary responses in TNCB-sensitized T cells *in vitro* **(Fig. 17)**. Comparing BMDC to cLC in their ability to present haptens, we found that these two cell types were equally efficient **(Fig. 17)**.

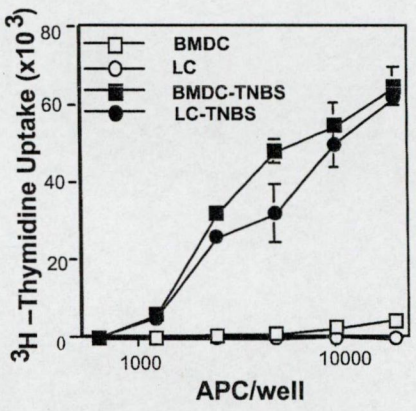


Figure 17. *In vitro* proliferative response of TNCB-sensitized T cells to TNBS-conjugated BMDC and LC. Mice were painted on the shaved thorax and abdomen with 3% TNCB. Cervical, axillary and inguinal lymph nodes were harvested 6 days later, purified for $CD4^+$ T cells and 2 x 10^5 cells/well of these responder cells were plated in a 96-well flat-bottom tissue culture plate. BMDC and 2-day cultured LC were conjugated with 1 mM TNBS for 10 min (BMDC-TNBS and LC-TNBS), washed extensively, irradiated and plated to achieve various responder:stimulator ratios. Non-conjugated BMDC and LC served as negative controls. TNBS-conjugated BMDC induced a response similar in magnitude to TNBS-conjugated LC. When cultured alone, T cells [non-stimulated or staphylococcal enterotoxin B (10 μg/ml) stimulated] as well as irradiated stimulators did not proliferate. This experiment is representative of two independent experiments.

4.8. Injection of TNP-conjugated DC induces contact hypersensitivity

We next determined whether BMDC were able to present haptenated antigens *in vivo*. We found that naive BALB/c mice injected with graded numbers of TNP-modified or unmodified BMDC developed a contact sensitivity response upon TNCB challenge. **Figure 18** shows that as few as 1 x 10^5 TNP-conjugated BMDC were able to induce a significant contact sensitivity response, comparable in magnitude to the responses generated by an equal number of TNP-conjugated LC. This finding is consistent with the *in vitro* results showing that there is no significant difference between BMDC and LC in presenting haptens.

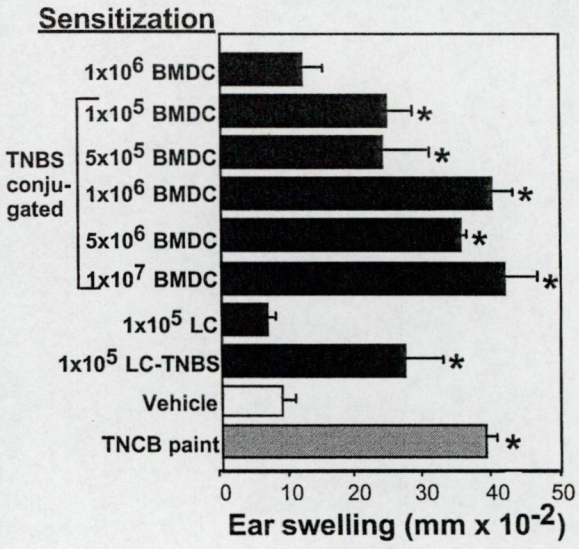


Figure 18. Injection of TNBS-conjugated BMDC induces contact sensitivity *in vivo*. BMDC and LC were incubated with 1 mM TNBS for 10 min (LC-TNBS), washed 3 times, injected in 100 μl HBSS/5% FCS s.c. (graded numbers of TNBS conjugated BMDC or 10^5 LC per mouse) into the dorsal skin of adult BALB/c mice. As a positive control, mice were sensitized by epicutaneous application of 100 μl of 3% TNCB to the abdomen. Mice were painted with the vehicle alone on the abdomen or alternatively injected with non-conjugated BMDC (negative control). Five days later, the right ears were painted with a 1% TNCB solution and ear thickness was measured 24 h later. TNBS-conjugated BMDC (10^5) were able to induce a significant contact sensitivity response, which was comparable in magnitude to the responses generated by an equal number of TNBS-conjugated LC. Experimental groups consisted of five mice each. $*P < 0.05$ assessed by two-tailed Student's *t*-test.

4.9. Heat-killed TNP-conjugated BMDC fail to induce contact sensitivity

To further investigate whether BMDC present the haptenated antigen themselves or whether shed antigens may be picked up and presented by host APC, we heat-killed the BMDC either before or after conjugation with TNP. The cells remained morphologically intact and >98% of the cells stained with the vital dye Trypan blue as revealed by microscopic examination. When these heat-killed cells were used there was no significant contact sensitivity response detected. **Figure 19** shows that even when large numbers (1 x 10^7) of TNP-conjugated heat-killed BMDC were used, contact sensitivity responses did not ensue.

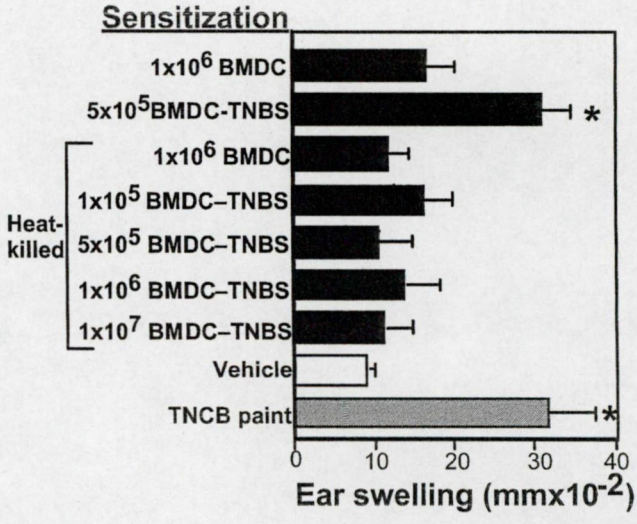


Figure 19. Heat-killed TNBS-modified BMDC do not sensitize for contact hypersensitivity. BMDC (3–5 x 10^5) were injected s.c. as unconjugated cells, TNBS-conjugated live cells (BMDC-TNBS, conjugation as described in Fig. 6.) or heat-killed dead cells (after conjugation cells were cultured at 56°C for 50 min; cell viability assessed by Trypan blue exclusion was <3%). Positive and negative controls were carried out as in Fig. 4. Increase of ear thickness was measured 24 h after challenge. Heat-killed TNBS-conjugated BMDC (10^7) were not able to induce contact sensitivity. Experimental groups consisted of five mice each. Two-tailed Student's *t*-test was used for statistical analysis.

4.10. TNP-modified BMDC induce contact sensitivity in an MHC-restricted manner as assessed *in vivo*

Thereafter, we injected the TNBS-conjugated or non-conjugated BALB/c- and C57BL/6-derived BMDC into parent and into F_1 mice. Results obtained in three independent experiments **(Fig. 20)** showed that the TNBS-conjugated BMDC induced contact sensitivity when injected into syngeneic mice and less, but still significant, contact sensitivity in F_1 mice. In contrast, if the cells were injected into allogeneic hosts, these mice were unable to develop a contact sensitivity response. Thus, we were able to show by *in vivo* assay, that BMDC present haptens and sensitize T cells in an MHC-restricted manner, and that, in keeping with

the results of experiments using soluble protein antigens, cross-presentation of haptenated antigens could not be detected.

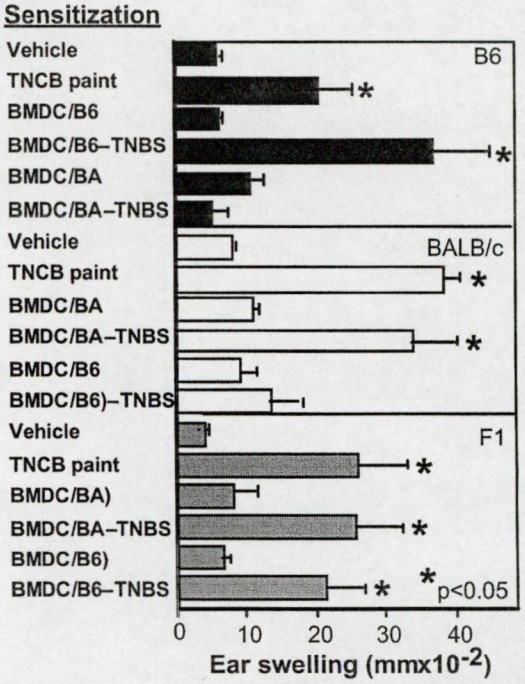


Figure 20. TNBS-conjugated BMDC induce contact sensitivity in an MHC-restricted manner *in vivo*. BMDC were harvested from BALB/c and C57BL/6 mice, conjugated with 1 mM TNBS for 10 min, washed 3 times, and 3–5 x 10^5 of these TNBS-conjugated (BMDC/BA-TNBS, BMDC/B6-TNBS) or non-conjugated BMDC (BMDC/BA, BMDC/B6) were injected s.c. in 100 µl HBSS + 5% FCS into the dorsal area of parenteral and F_1 adult mice. Mice were challenged 5 days later as described in Fig. 6 and ear thickness was measured 24 h later. Positive and negative controls were carried out as in Fig. 6. TNBS-conjugated BMDC sensitized syngeneic and, to a lesser extent, F_1 mice, but not allogenic mice. Each group contained five mice. The experiment shown is one of three independent experiments. One-way ANOVA was used for statistical analysis.

5. Discussion I.

In this study, using *in vitro* and *in vivo* functional assays we determined the efficiency of BMDC as APC for protein- and hapten-restricted responses. BMDC cultured with GM-CSF and IL-4 for 8 days resembled DC with a highly mature phenotype. If cells were pulsed with soluble protein antigen during maturation, they induced antigen-specific $CD4^+$ T cell proliferation *in vitro*, suggesting that they effectively processed and presented these antigens. Similarly, BMDC were able to present haptenated antigen to sensitized T cells *in vitro*, which is consistent with previous findings (77, 80).

We also showed that exogenous proteins and haptens are presented in an MHC-restricted manner; that protein or hapten-pulsed BMDC were not able to immunize allogeneic hosts; and that if parental (A) protein-pulsed BMDC were injected into F_1 (A x B) hosts, secondary T cell responses were detected only when T cells were re-stimulated with syngeneic parental (A) antigen-pulsed APC.

These findings demonstrate that haptens and proteins are not cross-presented to $CD4^+$ T cells *in vivo*, and are in contrast to the phenomenon that has been shown for peptides presented in association with MHC class I molecules. Furthermore, the findings suggest that although uptake of apoptotic (81) and necrotic (29) cells by immature DC has been demonstrated, functional cross-presentation might differ with MHC class II molecules compared to MHC class I molecules. The phenomenon of 'cross-presentation' [see review (41)], when antigens are transferred from cells or tissues and are represented on MHC class I and II molecules of APC, plays an important role in the maintenance of tolerance (70) and in the rejection of transplants (82) or rejection of tumor cells (83). It is known that immature DC present antigens from endocytosed particles, as shown for bacteria (84), apoptotic cells (81) and live cells (85). In a recent study Inaba *et al.* showed that processing of cellular fragments onto MHC class II may occur with both apoptotic and necrotic cells (29). Using Y-Ae, which recognizes a complex of $I\text{-}A^b$-presenting peptides from another MHC class II product, $I\text{-}E_{\alpha}$, they found large amounts of $Y\text{-}Ae^+$ cells in the T cell area of draining lymph nodes of C57BL/6 mice injected with live BALB/c-derived BMDC. These findings indicate that host APC picked up live injected BMDC either in the periphery or in the draining lymph node, therein potentially presenting peptides derived from the injected cells. Inaba *et al.* (86) have also shown this phenomenon and demonstrated that antigen-laden splenic DC can present antigen efficiently to unseperated lymph node T cells from mice with the same MHC

products but not from allogeneic mice. Furthermore, Smith *et al.* (87), found that despite the fact that peptide-pulsed $CD8^+$ murine splenic DC did not migrate to draining lymph nodes after footpad injection, they were able to stimulate a significant T_h cell response.

In our studies we used exogenous proteins and hapten, which were not previously assessed in cross-presentation studies, and our findings indicate that these antigens are not cross-presented to DC 4^+ T cells by host APC. Furthermore, in our studies, necrotic (heat-killed) antigen-pulsed cells were not capable of sensitizing the host, even when large numbers were injected, suggesting that although necrotic cells are capable of inducing maturation of APC and of initiating potent immunogenicity of DC, these types of antigens are not represented by the host. Based on our findings that BMDC required very high levels of soluble antigen to be able to prime naïve T cells or induce antigen-specific secondary T cell proliferation, the antigen presented on the cell surface might still not be sufficient for cross-presentation by host APC.

Recent findings showing cross-presentation of cell-associated OVA required 10 mg/ml of soluble OVA for pulsing spleen cells (88), a level significantly higher than the amount we used. In addition, cross-priming was shown to initiate CTL responses—there is little information about the initiation of CD4 responses. Using OT-I (CD8) and OT-II (CD4) TCR transgenic cell lines it has been demonstrated that cross-presentation of cell-associated OVA required 100-fold more OVA to stimulate $CD4^+$ cells from OT-II mice when compared to $CD8^+$ cells from OT-I mice (89). Although the authors emphasize that there is no way to determine whether the response of OT-I or OT-II cells reflects the strength of the response, or the ability to prime normal naive OVA-specific CD8 or CD4 cells, there may be a difference in the antigenic requirement for cross-presentation of MHC class I- and II-associated molecules. The level of antigen exposure may be increased as the number of APC increases. However, even when we injected as much as 10^7 cells per animal in order to expose the host to greater amounts of antigen we did not detect cross-presentation to $CD4^+$ T cells or significant DTH or contact sensitivity responses. Taken together, these experiments increase our understanding about the potential *in vivo* immunogenic effects of BMDC used for vaccination and immunotherapy protocols. They show that, although cross-presentation of cell-associated and soluble antigens has been demonstrated, allogeneic or heat-killed soluble antigen-loaded BMDC are not capable of priming CD4 T cells, indicating that cross-presentation of antigens presented in this way is inefficient.

CHAPTER II.

6. Results II.

6.1. CXCR5 is expressed in a migratory population of skin-derived DC

We compared the quantitative expression of CXCR5 and CCR7 in several DC populations **(Table 1)**: migDC, bone marrow-derived DC (BMDC), and fresh and cultured LC. LC cultured in an epidermal suspension for 3 days have an activated phenotype (90). CXCR5 expression in fresh LC was calculated to be approximately 50-fold lower than in migDC, whereas cultured LC expressed similar levels of CXCR5 compared to migDC. Since enrichment of I-A^d-positive cells from epidermal suspensions was necessary to observe CXCR5 expression by conventional reverse transcription (RT)-PCR (data not shown), it was unlikely that keratinocytes expressed CXCR5. By contrast, splenic B cells, which are known to strongly express CXCR5, showed an approximately 50-fold higher expression of this receptor than migDC **(Table 1)**. Thus, migDC and cultured LC have increased CXCR5 message levels relative to fresh LC, but lower message levels than B cells.

	Purity	CCR7 Expression relative to migratory DC	CXCR5 Expression relative to migratory DC
Splenic B cells	95%	33%	5100%
Fresh LC	78%	0.09%	2%
Cultured LC	89%	79%	45%
BMDC	90%	71%	7%
Migratory DC	90%	100%	100%

Table 1. Quantitative RT-PCR measurements of chemokine receptor mRNA expression in various DC populations. Splenic B cells, fresh LC, cultured LC, BMDC and migDC were isolated as described Methods. Expression is displayed relative to migDC (= 100 %). This single experiment was performed using means of duplicate samples and is representative of three separate experiments using semiquantitative RT-PCR methods.

6.2. MigDC, but not BMDC, respond chemotactically to BLC *in vitro*

To demonstrate that CXCR5 mRNA expression correlated with function in migDC, we performed chemotaxis assays using recombinant murine BLC as a stimulus and migDC and BMDC as targets. As shown in **Fig 21 a,** BLC stimulated chemotaxis of DC approximately 4-fold over baseline. Concentrations of BLC above 625 ng / ml (*i. e.* 1,000 ng / ml) did not enhance migration of migDC. BLC was not as efficient as SDF-1 or SLC in inducing chemotaxis **(Fig. 21 b)**. By contrast, BMDC did not respond to BLC, but did

respond to SLC with an 8-fold increase in migration **(Fig. 21 c)**. By phase microscopy, > 95 % of all cells in BLC-stimulated wells were dendritic in morphology and were indistinguishable from SLC-stimulated or non-stimulated cells (not shown). Flow cytometric analysis revealed that 98 % of all cells that had migrated to BLC were MHC class II^{high} in a pattern indistinguishable from migDC that had migrated to SLC (not shown). Furthermore, the cells that migrated to BLC did not show either specific B220 or CD3e staining (similar to SLC-stimulated cells). Thus, the cells that migrated in response to BLC were neither B cells nor T cells and appeared to have classic morphological and surface phenotypic characteristics consistent with DC.

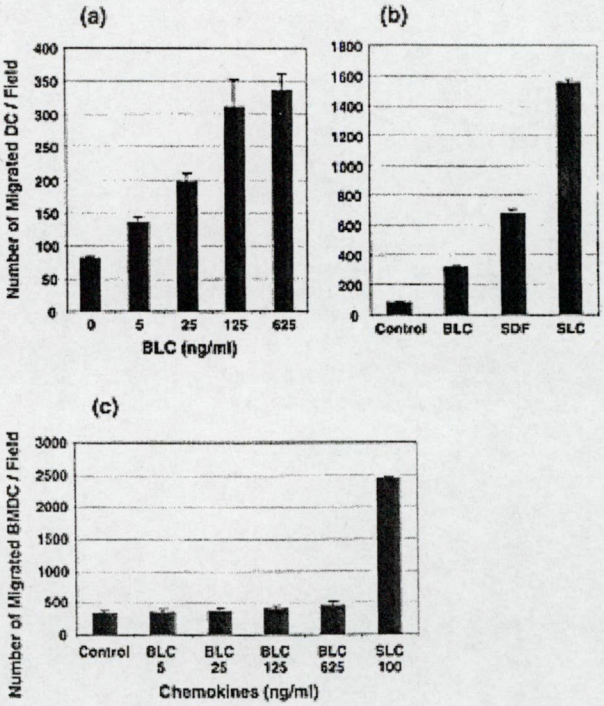


Figure 21. MigDC respond to BLC *in vitro*. Cultured migDC were used in microchemotaxis assays with recombinant murine BLC, SLC, and human SDF-1 as chemoattractants. (a) Dose response of migDC to BLC, (b) response of migDC to 100 ng / ml BLC, SDF-1, and SLC, and (c) response of BMDC to BLC and SLC. Optimal migDC migration to SDF-1 and SLC were achieved at 100 ng / ml in preliminary experiments. Results represent one of at least three experiments with similar results. Results are shown as mean + SEM cells per field averaged from four fields per treatment condition.

6.3. MigDC migrate to B, as well as T, cell areas of the LN *in vivo*

To address the question of whether expression of CXCR5 directed the migration of DC to specific areas of LN, we recovered migDC from skin, labeled the cells with a stable membrane dye, and injected them into the footpad of mice. After 24, 48, and 72 h, popliteal LN were removed and stained with a B cell marker (CD45R / B220, **Fig.22 a**) or a T cell marker (CD3e, **Fig. 22 c**) to delineate complementary B and T cell-rich areas. MigDC migrated to B cell zones as shown at low magnification in Fig. 20 c (white square region) and at high magnification in Fig.20 d. MigDC were also observed at the boundary zones between B and T cell zones [**Fig. 20 c** (red square region), e]. The majority of migDC were found in T

cell zones **(Fig. 22 f)**. Not all B cell follicles in the LN contained visible DC. MigDC were also detected in subcapsular spaces (not shown). By contrast, BMDC localized exclusively to T cell areas (not shown), as reported recently (91). To demonstrate that membrane labeling of the DC did not substantially alter the migration pattern of migDC, we also injected unlabeled migDC and BMDC into the footpad of mice. When LN from noninjected mice were sectioned and stained for CD11c, positively stained dendritic-appearing cells were found predominantly in T cell-associated areas and not in B cell areas (not shown). After injection of BMDC, $CD11c^+$ DC again appeared to be largely excluded from B cell areas (not shown). By contrast, after injection of migDC, $CD11c^+$ cells were visible in B cell-associated zones in a pattern consistent with that observed with labeled migDC **(Fig.22 g, h)**. Thus, migDC appeared to migrate to B as well as T cell areas, whereas BMDC migrated almost exclusively to T cell areas.

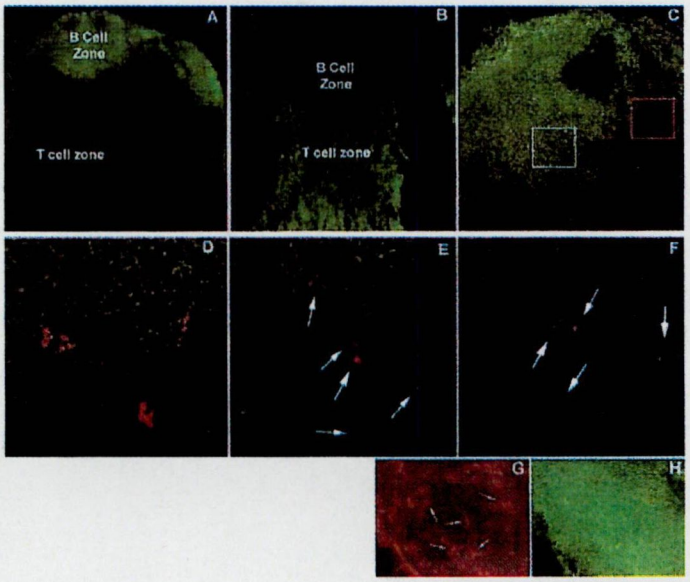


Figure 22. *In vivo* homing of fluorescently labeled migDC to distinct regions of LN. (a - b) Sections of mouse LN were individually stained with FITC-conjugated anti-CD45R / B220 (a) and FITC-conjugated anti-CD3e (b) to demonstrate staining of complementary B and T cell regions. (c - f) MigDC were labeled with PKH26 (red) membrane dye and injected into the footpad of mice. Animals were killed 24, 48, or 72 h later, and the popliteal LN draining the injected footpad were removed and frozen sections obtained for two-color confocal microscopy. All the photos in (c - f) were taken from the 48-h time point. (c) MigDC (red) within B cell zones (stained green with FITC-conjugated anti-CD45R / B220) as well as T cell zones (20 × objective). (d) The white square region within the B cell zone in (c) was imaged with a 60 × objective to demonstrate individual migDC in red. (e) A border region betwen B and T cell zones [illustrated by the red square in (c) above] showing migDC, 60 × objective. (f) MigDC in a non-B cell (presumably T cell) zone, 60 × objective. (g, h) Unlabeled migDC were injected into footpads. Sections from the draining popliteal LN were double-stained with anti-CD11c (g, MigDC in red) or CD45R / B220 (h, green) and imaged separately with red (g) and green (h) filters by conventional immunofluorescence microscopy, 20 × objective. Arrows indicate DC.

7. Discussion II.

Our finding that activated migDC express CXCR5 and respond to BLC provides the first physiologically relevant mechanism for the migration of DC to B cell areas. Just as mature DC and naive T cells are brought to LN via mechanisms related to CCR7 (57, 62) DC and B cells may utilize the same chemokine receptor, CXCR5, to find their way to B cell zones. However, because of the lack of suitable blocking reagents, we have not been able to determine whether BLC and CXCR5 are absolutely required for migDC trafficking to B cell areas; it is quite possible that other chemokines and chemokine receptors may also play a role in this process. Some DC populations (*i. e.* migDC) appear to express CXCR5 at sufficient levels to respond to BLC, whereas others (*i. e.* BMDC) do not. This may explain why several studies with splenic (52) and bone marrow-derived (91) DC found migration of these particular DC to T cell zones of LN.

Of note, migDC did not appear to be distributed in all B cell follicles. Interestingly, while BLC was found to be expressed in most B cell follicles of spleen, BLC was variably expressed in B cell follicles in peripheral LN (57). Thus, the pattern of migration we observed with migDC may reflect the higher expression of BLC in some of the B cell follicles of the LN.

We have shown that skin-derived migDC up-regulate CXCR5 as they become activated and leave the skin in an *ex vivo* model of DC trafficking. MigDC functionally respond to BLC, which has previously been described exclusively as a B and (weak) T cell chemoattractant. *In vivo* this population of DC migrated from the footpad to regional LN, and up to 40 % of labeled migDC localized to B cell areas, although migDC were also apparent in T cell zones. Based on the strong expression of CCR7 by migDC, we propose that migDC initially utilize CCR7 to migrate to afferent lymphatics and / or SLC / ELC-rich T cell areas before migrating to B cell areas via CXCR5. Once in B cell areas, migDC may have direct effects on B cells as data from others have shown. Thus, the regulated expression of CXCR5 on certain populations of DC may play a role in determining the migration pattern of DC within secondary lymphoid organs.

CHAPTER III.

6. Results III.

6.1. Labeling with F-18 has no significant effect on the ability of BMDC to induce allogeneic T cell proliferation or to migrate in an in vitro chemotaxis assay

Figure 23 a shows that when either F-18 labeled BMDC or control sham-treated BMDC were cultured for 5 days with allogeneic T cells, both populations induced similar levels of secondary proliferation of T cells, indicating that labeling did not affect the ability of BMDC to induce a mixed leukocyte reaction. It has been shown that mature BMDC express CCR7 (92) the receptor for the secondary lymphoid tissue chemokine (SLC), and that these cytokines play an important role in the trafficking of DCs from the periphery to the regional LN in vivo (62). When F-18-labeled BMDC were compared in their ability to migrate towards SLC in trans-well chambers, there was no significant difference between the F-18 labeled BMDC and the sham-treated BMDC **(Fig. 23 b)**.

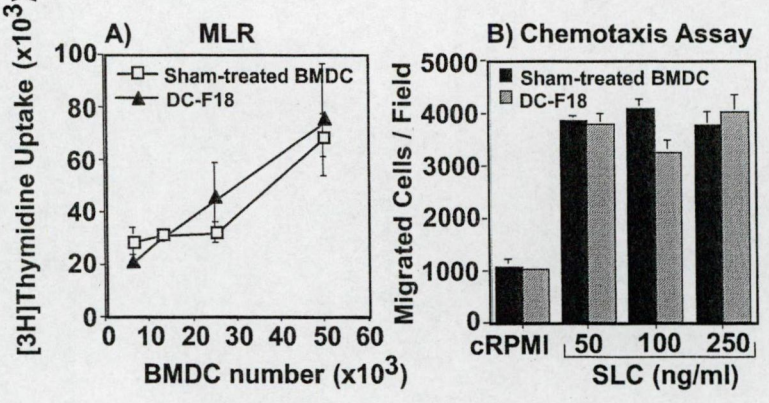


Figure 23. F-18 labeled BMDC function similarly to sham-treated BMDC in an allogeneic mixed leukocyte reaction and in an in vitro migration assay. Cells were labeled with F-18 (closed triangle) or were sham-treated (open square). (A) Twenty-four hours later, cells were γ-irradiated with 3000 rad, and added in graded numbers to 2×10^5/well allogeneic C57BL/6-derived naive T cells. BMDC number was normalized by the percentage of class II^+ cells by flow cytometry. Cell proliferation was measured on day 5 by counting [^{3}H]thymidine (mean±SE in triplicate wells) incorporation in the last 16 h of culture. Representative data from one of three similar experiments are shown. In the chemotaxis assay (B) BMDC were labeled with the intracellular fluorescent dye, calcein-AM at 1 μM for 30 min at 37 °C, washed extensively and subsequently labeled with F-18 (hatched bar), as described in , or with PBS (sham-treated BMDC—closed bars). A total of 2.5×10^4 cells were loaded on the top of the filter. Murine SLC was placed in different concentrations in the bottom well to attract BMDC. After 4 h at 37 °C in a humidified incubator, the cells in the bottom of the chamber were enumerated in four randomly chosen 10× views and averaged. Results are expressed as the mean±SEM for duplicate wells.

6.2. BMDC migrate to the T cell areas of draining LN

To confirm the migration capability of our BMDC, we labeled the cells with a stable membrane dye, and injected them into the footpad of mice. After 48 h, popliteal LN were removed and frozen sections were observed with a fluorescent microscope. BMDC migrated to the paracortical areas and subcapsular sinuses (T cell areas) shown at low magnification in **Fig. 24 b**, **Fig. 24 a** shows the same area under phase contrast microscope. To demonstrate that the observed fluorescence was associated with the injected cells and was not due to ingested dye from host migratory cells, we injected large numbers (1×10^7) of heat-killed PKH67-labeled BMDC into the left hind footpad. After 48 h we were not able to detect any specific fluorescently staining cells in the draining popliteal LN as shown in **Fig. 24 d.**

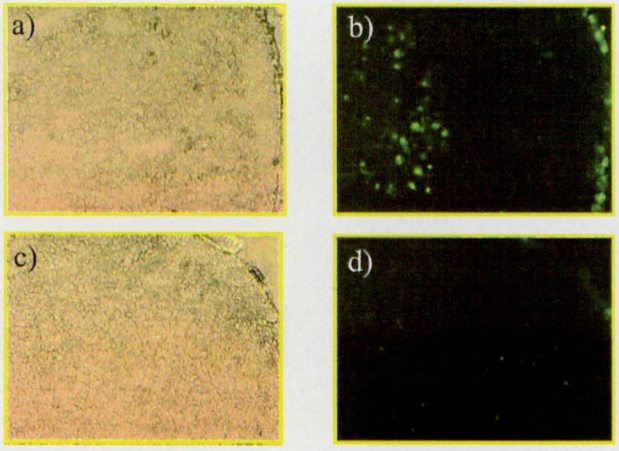


Figure 24. Fluorescently labeled BMDC migrate to T-cell regions of LN. Live (a, b) and heat-killed (c, d) BMDC were labeled with PKH67 (green) membrane dye and injected into the footpads of mice. Animals were killed 48 h later, and the popliteal LN draining the injected footpad were removed and frozen sections obtained for fluorescent microscopy. Live PKH67-labeled BMDC were found in the parcortical area and in the subcapsular sinuses (b), while heat-killed PKH67-labeled BMDC could not be detected in the draining lymph node (d).

6.3. Positron imaging of F-18-labeled BMDC with PiPET

Figure 25 shows the whole-body projection image of a mouse imaged with PiPET 4 h after footpad injection with F-18-labeled BMDCs (after removal of the bladder). The footpad contains, by far, the largest amount of activity in this image and is overexposed to allow lesser amounts of activity elsewhere in the body to be seen. The popliteal LN is readily visible by virtue of the BMDCs being intensely concentrated in a very small structure. The

LN and the kidneys appear in their correct anatomical location. An additional accumulation of activity is also evident in the liver region. The ratio of net popliteal lymph node activity to net footpad activity determined from these images averaged 0.15% for the two animals, a value that compared well with ratios determined by tissue assay; average of 0.18%. Activity found in the lymph node as the percentage of the activity in the footpad was calculated as follows: (LN/FP ratio)=[Area×(Mean brightness-Background brightness)/Area of FP×(Mean brightness-Background brightness)]×100.

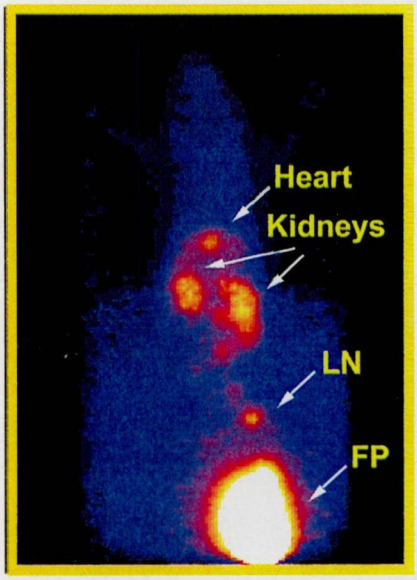


Figure.25. PiPET image of a whole mice injected with F-18 labeled BMDC into the footpad. 5×10^6 F-18 labeled mature BMDC were injected into the left hind footpad of naive BALB/c mice and 4 h later the mice were anesthetized and the bladder was removed. The mice were imaged for 10 min with the PiPET camera. Activity is found in liver, kidneys, draining popliteal lymph node and injected footpad.

7. Discussion III.

This is the first description of a method for labeling mouse BMDC with a positron emitting radionuclide F-18 in order to follow their migration with a high-resolution imager in vivo. F-18 is a radionuclide with a short (109.7 min) half life, and a high specific activity (>200 Ci/mmol at EOB). The short half life allows detection of the radionuclide for a maximum of 5–6 h, which in the case of BMDC migration is rather short, since the maximum number of injected BMDC can be found in the draining lymph node after 48 h in mice (91). However, because of the availability of F-18 and its ability to be detected by PiPET, we decided to use this radionuclide for determining the possibility of monitoring in vivo migration.

There have been several studies indicating the detrimental effects of isotope labeling of lymphocytes (93, 94);however, more recent studies using either In-111 (95) or ^{111}In-oxine, ^{18}F-FDG and ^{99}mTc-HMPAO (96) report acceptable toxicity and potential uses of these labeling methods.

Dendritic cells have been labeled with other radionuclides (62, 73, 97, 98); however, we are not aware of any studies using F-18 labeled BMDC. Therefore, it was crucial to determine the effect of the radiolabeling on these cells, in order to be able to use them in in vivo studies. Due to the short half life of F-18, our aim was to determine the short-term (max. 24 h) effect of the labeling. Labeling with F-18 had no significant effect on the viability or the phenotype of BMDC compared to sham-treated control cells, suggesting that these cells are fairly resistant to gamma and positron radiation. Furthermore, using an in vitro chemotaxis assay we found no difference in the capacity of these cells to migrate in vitro compared to non-labeled DC. The short half life of F-18 did not allow us to perform long-term in vivo experiments; however, even at 4 h we could detect significant cell-bound activity in the draining lymph node. It was critical to determine whether the detected activity was related to cells or was free activity released by the labeled cell, because of the large amount of activity F-18 labeled cells released during in vitro culture. We tried to determine whether this release was passive shedding of membrane-bound activity or was related to cell metabolism, and found that release was significantly reduced if cells were kept at low temperatures in order to reduce their metabolic activity. The exact mechanism of this release requires further investigation. Better maintained radioactive labeling agents are also currently under development. However, using F-18 labeled macrophages, F-18 labeled dead BMDC or F-18

labeled 4-fluorobenzoic acid for injection confirmed that the activity found in the draining lymph node after BMDC-F-18 injection represented cell-bound activity, since less activity was found in the case of controls. We chose footpad injections to establish the new method, based on previous in vivo migration experiments with fluorescent dye labeled BMDC. Previous studies in mice (97) and in chimpanzees (72) showed that after IV injection, DC accumulate first in lungs then in liver and spleen, organs in which injected cells are likely to lodge nonspecifically.

PiPET imaging offers the possibility of studying in vivo cell migration, because radiolabeled cells can be detected in live animals. This method enables us to manipulate the animal during imaging, for example, to study the effects of certain chemokines or inflammatory agents on the migration of the radiolabeled cells. Furthermore, this camera gives a ~2 mm spatial resolution, and has the potential to produce a 3D image. Using image analysis the exact amount of radioactivity can be determined and extrapolated to the number of cells. Furthermore, background activity can be subtracted to produce accurate quantitative measurements. Certain technical issues such as the proper anesthesia for mice while in the PET camera, and the effect of anesthetics on the migration need careful consideration.

Our next goal is to develop a radiolabeling method with minimum loss of activity and maximum half life in order to be able to study the effect of certain anti-inflammatory agents on the migration pattern of DC. This method currently provides the possibility of in vivo imaging with high accuracy and offers a new approach for in vivo studies of dendritic cell biodistribution and migration.

8. Summary

- **Murine BMDC are useful for studying cell-based immunization**
- **Cultured epidermal Langerhans cells express higher levels of costimulatory molecules compared to BMDC and require less soluble antigen to induce antigen specific secondary immune response**
- **BMDC present soluble protein antigens and haptenated antigens in vitro and vivo**
- **Soluble protein antigen and haptenated antigen presentation to $CD4^+$ T cells is MHCII-restricted**
- **Host antigen presenting cells do not cross present soluble antigens and haptens presented on allogeneic BMDC**
- **In vitro cultured antigen pulsed and non-pulsed BMDC migrate to the regional LN with the same efficiency**
- **Migratory DC express 50-fold more CXCR5 mRNA than fresh Langerhans cells**
- **Migratory DC migrate in response to B lymphocyte chemoattractant *in vitro***
- **When injected into the footpad of mice, migratory DC emigrate to the B and T cell area of the regional LN**
- **We developed a novel cell-labeling method using the positron emitting radionuclide F18**
- **F18-labeled BMDC are phenotypically and functionally similar to non-labeled BMDC**
- **PiPET imaging of F18-labeled BMDC offers a new approach for in vivo studies of dendritic cell biodistribution and migration**

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Appendix

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