Synthetic lipid A mimetic adjuvants stimulate the maturational and functional development of murine bone marrow derived dendritic cells

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Synthetic Lipid A Mimetic Adjuvants
Stimulate the Maturational and Functional Development of
Murine, Bone Marrow Derived Dendritic Cells

By

Kevin Floyd

B.S. Rutgers University, New Brunswick, New Jersey, 1987

Presented in partial fulfillment of the requirements for the degree of

Master of Science

The University of Montana

2001

Approved by:

[Signatures]

Chairman, Board of Examiners

Dean, Graduate School

7-30-01

Date
Lipid A Mimetic Adjuvants Stimulate the Maturational and Functional Development of Murine, Bone Marrow Derived Dendritic Cells. (54 pp.)

Committee Chair: George L. Card, Ph.D.

Dendritic cells (DCs) are considered the only 'professional' antigen-presenting cell (APC) capable of educating naïve T cells. Ex-vivo manipulation of autologous, immature DCs for antigen loading and induced maturation for re-infusion back into the patient as potent APCs has been considered a plausible approach for cancer immunotherapy (Fong et al. 2000. Annu. Rev. Immunol. 18:245-273.). Major histocompatibility (MHC) class II expression, CD40 and co-stimulatory molecules, CD80 and CD86 (which are important in T cell receptor (TCR) engagement and APC/T helper and cytotoxic cell interaction) are upregulated upon maturation of human and murine bone marrow derived DCs (bmdDC) (Labeur et al. 1999. J. Immunol. 162:168-175.). Lipopolysaccharide (LPS) is one such maturation agent that has been shown to induce the phenotypic maturation of bmdDCs helping them to stimulate therapeutic tumor immunity (Labeur et al.).

Corixa's (Seattle, WA.) proprietary synthetic lipid A mimetic compounds RC511, RC527, RC529, and RC544, previously screened as adjuvant candidates for vaccine formulations, were tested in comparison to monophosphoryl lipid A (MPL) and the parent compound for MPL, R595 S. minnesota LPS, for their ability to induce functional maturation of murine bone marrow derived DCs (bmdDCs). Murine bone marrow-derived DCs were grown in 10 day in-vitro cultures and subsequently pulsed in-vitro with ovalbumin. Antigen loaded DCs were then stimulated in-vitro with MPL, R595 LPS, and with RC511, RC527, RC529, and RC544 to promote functional and phenotypic maturation of the DCs. Antigen-loaded, mature DCs were then used as putative APCs in an in-vitro assay utilizing ova-specific naïve CD4\(^+\) splenic T cells from the ova-TCR (T cell receptor) specific transgenic DO11.10 (Balb/c background) mouse system as effector T cells. All CD4\(^+\) T cells in the murine DO11.10 model have the transgene for a TCR complex specific for the ovalbumin peptide 323-339 bound to I-A\(^d\), class II, MHC molecules. Lipid A maturation of antigen pulsed, bmdDCs at the 100ng/ml dose induced very significant increases of secreted cytokines in the DO11.10 functional assay at the 10:1 T cell:DC ratio as measured by ELISA. This included increases from several hundred percent for IL2 to several thousand percent for IFN-gamma and IL-12p40 above the ovalbumin-only pulsed, lipid A induced bmdDC controls. MPL-S\(^®\), RC527, RC529 and RC544 showed a dose range effect between 10 and 500ng/ml in the levels of secreted IFN-gamma, IL-12 p40 and to a lesser degree IL2. Experimental data suggested RC511 and RC527 were optimally effective at stimulating cytokine secretion when used at doses as low as 10ng/ml in-vitro.

The cumulative data from this study shows that all the synthetic lipid A compounds except RC529 had very potent ability to stimulate phenotypic maturation of cultured DCs as evidenced by the dramatic cell surface up-regulation of I-A\(^d\) (MHC class II), CD40, CD80 and CD86 molecules (which are important in T cell receptor (TCR) engagement and APC/T helper and cytotoxic cell interaction). These synthetic lipid A molecules also uniformly (with the exception of RC529) stimulated ova-pulsed DCs to drive high levels of T cell activation as evidenced by the antigen-specific, DC-dependent T cell secretion (DO11.10 T cells) of the Th1 cytokines IFN-gamma and IL-2. The levels of phenotypic and functional maturation stimulated by the lipid A mimetics was on average equal to or higher than that induced by natural MPL (at the same concentration; 100ng/ml).
ACKNOWLEDGEMENTS

I would like to first thank my parents for instilling in me the persistence and drive to overcome the obstacles necessary to accomplish this thesis work. I would like to sincerely thank Dr. Edwin Walker for his pursuit of scientific truths, passion for immunological research and encouragement at difficult junctions. I would also like to thank my other committee members for their expertise and valuable time; Dr. George Card for his lasting impression of dedication, patience and integrity and Dr. Mike Minnick for his excellent teaching ability and enthusiasm for immunology. I would also like to thank my former, fellow employees and researchers at Corixa in Hamilton, MT. (formerly Ribi ImmunoChem Research, Inc.) who helped make possible my completion of this thesis project and fostered my learned passion for exploring and discovering something that could truly, possibly benefit humankind.
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<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<td>DC</td>
<td>Dendritic Cell</td>
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<td>ELISA</td>
<td>Enzyme Linked Immunosorbant Assay</td>
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<td>ETOH</td>
<td>Ethanol</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate, a fluorescent tag for antibodies</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor which binds the Fc portion of antibodies</td>
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<td>G</td>
<td>G force</td>
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<td>IL</td>
<td>Interleukin, cytokine released from leukocytes</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MCF</td>
<td>Mean Channel Fluorescence</td>
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<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>MPL-S</td>
<td>Monophosphoryl Lipid A, S formulation</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>RC</td>
<td>Ribi Compound</td>
</tr>
<tr>
<td>rmGM-CSF</td>
<td>recombinant murine Granulocyte Macrophage- Colony Stimulating Factor</td>
</tr>
<tr>
<td>TC</td>
<td>T cell</td>
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<tr>
<td>TGFB₁</td>
<td>Transforming Growth Factor- Beta</td>
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<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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CHAPTER 1
INTRODUCTION

Dendritic cell heterogeneity and phenotype

Dendritic cells (DC) have the unique ability to activate naïve T cells in the generation of primary T cell responses, thus allowing the establishment of immunological memory (1). Generally speaking, in both the mouse and human, four stages of development have been described. These stages of DC differentiation are somewhat characterized by their anatomic location. They include: (a) bone marrow progenitors; (b) precursor DCs that police the blood and lymphatics for pathogen recognition; (c) tissue residing immature DCs, which possess high potential for antigen (Ag) capture; and (d) mature DCs, present within secondary lymphoid organs, that express high levels of costimulatory molecules as well as MHC class I and class II molecules permitting efficient antigen presentation (2). In mice two distinct DC precursors, “myeloid” and “lymphoid” derived have been identified. Mouse DCs expressing CD8-alpha with low or null expression of CD11b are generally considered to belong to the lymphoid lineage, whereas CD8-alpha negative, CD11c⁺ and CD11b⁻ DC are described as myeloid lineage cells (3,4). In the human at least three subsets of DC precursors are transported in the blood: CD14⁻CD11c⁻CD1⁻ monocytes, CD14⁺CD11c⁺CD1⁺, and lineage-negative (i.e. not of T or B cell origin) CD11c⁻IL-3R-alpha⁺ precursor DCs (2). Growth factor conditions in-vitro can determine phenotype and sometimes functional outcome of DC differentiation. In humans, myeloid CD34⁺ progenitors (obtained from cord blood or bone marrow) can differentiate into CD14⁺CD11c⁺CD1⁻ monocytes, which can then grow into immature ‘interstitial’ DC in response to granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. Alternatively, when cultured in macrophage-colony stimulating factor (M-CSF) this same blood precursors yield cells with an ‘interstitial’ macrophage phenotype. Moreover, CD14⁻CD11c⁺CD1⁺ DC blood precursors grown in the presence of TGF beta in the GM-CSF/IL-4 culture yields Langerhans DC (LCs) (2). In mice myeloid-committed precursors from bone marrow, when grown in-vitro in the presence of only GM-CSF, yield both granulocytes/monocytes and myeloid DCs (5). In the mouse DCs can also arise from lymphoid precursors (6). The level of heterogeneity of human DCs in-vivo is represented by their anatomic location which includes skin epidermal LCs, dermal DC, splenic marginal DCs, T-zone.
interdigitating cells, germinal-center DCs, thymic DCs, liver DCs and blood DCs. Their lineage origins, maturation stages, and functional differences, however, have not been clearly established to date despite some observable phenotypic differences (2).

The role of costimulatory molecules in dendritic cell phenotype and function

As immature cells residing in non-lymphoid tissues, DCs can very efficiently capture antigen by phagocytosis and macropinocytosis (7). Subsequent to various modes of induction by, for example, CD40 ligand, TNF-alpha or lipopolysaccharide (LPS) the immature DCs start a maturation process that includes a significantly decreased ability for antigen uptake and a concomitant enhanced cell surface expression of MHC processed antigen and co-stimulatory molecules (8,9,10). For optimal activation of T cells, recognition of antigen/MHC complexes by the T cell receptor must be accompanied by a second, costimulatory signal found on an antigen presenting cell such as the DC. CD80 (B7-1), CD86 (B7-2) (11) and CD40 on the cell surface of antigen presenting cells such as DC fulfill this co-stimulatory function and stimulate increased levels of T cell activation as measured by both IL-4 and IFN-gamma secretion. None of these costimulatory molecules, however, appears to selectively regulate T cell helper 1 or 2 differentiation nor do they have an obligatory role in priming for the production of either T cell effector cytokines. CD86, perhaps having a more predominant costimulatory role than CD80, has been demonstrated in human peripheral blood dendritic cells. CTLA-4Ig and CD86 monoclonal antibodies (mAb), but not CD80 mAb, block an allogeneic mixed lymphocyte reaction (MLR) when stimulated by human peripheral blood dendritic cells that are initially negative for CD80 and CD86 before activation by mitomycin C (12).

In humans, cross linking the CD40 receptor on DCs induces increased expression of CD80 and CD86 on GM-CSF and TNF-alpha-differentiated dendritic Langerhans cells (13); similarly, CD40 receptor cross linking on DC-like (GM-CSF and IL-4-differentiated) blood mononuclear cells also stimulates increased CD80 & CD86 expression. (14). Cross-linking of CD40 directly on isolated lineage negative human blood DC with mouse trimer CD40L (mCD40LT) for 40 hours also markedly augments CD80 and CD86 upregulation (15). Moreover, when blocking antibodies CD40Ig and CTLA-4Ig (for blocking CD80 and CD86) are added simultaneously only minimal and variable additional inhibition of DC-stimulated allogeneic T lymphocyte proliferation and IL-2 secretion was observed compared to each antibody alone.
This suggests that both CD80/CD86-dependent and independent components of DC-T lymphocyte CD40:CD40 ligand co-stimulation exist and further emphasize that the majority of blood DC have to differentiate or be activated to express the co-stimulatory molecules” (15).

Role for CD40 receptor on DCs and CD40 ligand from T cells in cellular activation

DCs can also mediate CTL stimulation through presentation of antigen in the context of MHC class I restriction elements and the costimulation mediated by engagement of the CD40 receptor on the DC with the CD40 ligand found on the CD8+ T cell. CTL effectors probably act alone when killing target cells. Their differentiation from naïve CD8+ T cells may be fundamentally dependent on ‘help’ from CD4+ helper T (Th) cells (16,17). This CD4 mediated helper T cell function may be provided in a cognate manner, such that MHC class I and MHC class II restricted antigen epitopes are recognized by both the T helper cell and the CTL on the same antigen presenting cell (18). Alternatively, data also support the concept of initial DC mediated “priming” by CD4 helper T cell stimulation followed by CD8 activation by CD4 stimulated DCs presenting processed antigen in the context of MHC class I molecules. Further evidence suggests that signaling through CD40 can replace CD4+ T-helper cells in the priming of helper-dependent CD8+ CTL responses and that CD40-CD40L interaction is critical to the messaging of T-cell help for CTL priming. Support for this theory is that in the absence of CD4+ host cells addition of CD40 antibodies after immunization restores CD8+ CTL response by the host suggesting that CD40 can replace CD4+ T helper cells in the CTL priming via activation of the antigen presenting DC (19,20). This cross-priming phenomenon can sometimes occur when exogenous antigen is taken in and is processed and presented via MHC class I, in addition to the expected MHC class II presentation pathway (21). Recently, other investigators have found that in addition to a CD40-dependent pathway of T cell help there exists a CD40-independent pathway of T cell help for priming CD8+ cytotoxic T lymphocytes which still involves DC “sensitization” by antigen stimulated CD4+ helper T cells (22).

The ability of the CD40 ligand to stimulate functional, differentiated, bone-marrow-derived DCs, and promote antitumor protective and therapeutic (8) immunity in-vivo correlates with the concomitantly high CD40 ligand-mediated DC production of IL-12 p70 (8), which is known to favor T helper cell type 1 responses subsequent to antigen presentation by both mouse and human DCs (23). These observations are in agreement with in-vitro data obtained by others (24, 25) and may be a function of the culture conditions
the DCs were grown in, the length of time and type of agent used for maturation, and the type of tumor antigen tested (poorly immunogenic or not). It is notable that despite comparable maturation stages (as evident by down-regulation of phagocytosis, low endocytic capacity, and up-regulation of MHC class II, the adhesion molecules CD11c and ICAM-1, and the costimulatory molecules CD80 and CD86) LPS-stimulated DC were less capable of promoting tumor immunity than CD40L-treated bone marrow derived DC (8).

**Regulation of DC maturation and APC function**

The DC maturation process most likely starts after, or is concomitant with, antigen uptake and continues through the DCs' migration to the secondary lymphoid organs. This maturation process can be induced and/or be regulated by a multiplicity of factors. They include pathogen related substances such as lipopolysaccharide (LPS), bacterial DNA, double-stranded RNA; the net balance between pro-inflammatory and anti-inflammatory molecules in the local milieu including TNF, IL-1, IL-6, IL-10, TGF-β and prostaglandins and T-cell derived signals including CD40 ligand, IFN-gamma and IL-2 (2). Physical changes that appear with DC maturation include loss of adhesive cell surface molecules, cytoskeleton reorganization and acquisition of high cellular motility (9). Confocal microscopy of continuously passaged, immature murine splenic DC has revealed storage of high levels of MHC class II molecules in discrete intracellular vesicular compartments. Immature DCs are very efficient at antigen capture via several pathways such as (a) macropinocytosis; (b) receptor-mediated endocytosis via C-type lectin receptors (mannose receptor) or Fc-gamma receptor types I and II and (c) phagocytosis of particles such as latex beads, apoptotic and necrotic cell fragments. Upon maturation of the DC concomitant events occur which promote increased migration, heightened and enduring antigen presenting capacity, but also a profound correlated refractoriness to additional antigen uptake and processing. There is also a loss of phagocytic receptors (endocytic), upregulation of co-stimulatory molecules CD40, CD80, CD86, and change in morphology manifested by veiled-like appearance and a significant upregulation of cell surface class II MHC expression.

LPS and locally produced paracrine or autocrine TNF-alpha or IL-1 (26) all can induce DC maturation, and cause DCs from the periphery to migrate into the T cell areas of lymphoid organs. Immature DCs express high concentrations of chemokine receptors CCR1, CCR5 and CCR6. After a
maturation signal has been received they subsequently express lower concentrations of these receptors.

Subsequent to stimulation with maturation signals, the chemokine receptor, CCR7 is elevated on DCs with an acquired responsiveness to MIP-3-beta (27). This maturation process most likely continues upon DC and T cell interaction. DC-TC interaction mediated via CD40 receptors on DCs and CD40 ligand on T cells augment the upregulation of co-stimulatory molecules CD80 and CD86 on DCs.

In addition, interferon (IFN)-gamma, produced by T helper-1 cells and mediated by DC-derived IL-12 p70 (22), is also implicated in the maturation of the DCs. CD4+ T cell-induced IL-12 p70 produced by human peripheral blood derived DC requires two signals, CD40 engagement and IFN-gamma production (28). The inability of naïve T-helper (CD4+ CD45RO') cells to induce DC derived IL-12 p70 production comes from their inability to produce IFN-gamma. However, initially priming of the naïve T cells with the addition of IFN-gamma in the culture (in-vitro) subsequently helps produce significantly greater amounts of IL-12p70 by DCs compared to DCs which are in the presence of naive T cells that have been only stimulated by superantigen, staphylococcal enterotoxin B (SEB).

Besides the combination of CD40 ligand and IFN-gamma other factors such as LPS can supply the required signals for IL-12 p70 production by DC. A two signal requirement comprised of CD40 ligand and IFN-gamma is particularly important for IL-12 p70 since other pro-inflammatory cytokines like TNF-alpha, IL-8 and p40 subunit of IL-12 only require either CD40 engagement or LPS. CD40 engagement alone, however, cannot induce the production of biologically relevant IL-12 p70 by DCs (28). But since LPS has been shown to upregulate DC expression of CD40 receptor, MHC class I and II, and co-stimulatory molecules such as CD80 and CD86 and even adhesion molecule I-CAM there is perhaps a greater likelihood of sustained DC and T-cell interaction and T cell activation. Subsequently, the secretion of IFN-gamma by CD4+ helper and CD8+ T cells may yield more IL-12 p70 production as well.

**Deficient immunogenicity of tumors and major histocompatibility presentation of tumor antigens**

It has been demonstrated in various animal models that DC's have the ability to prime T cells to tumor specific antigens which subsequently are capable of proliferating, recognizing and killing tumor cells in an antigen-specific fashion (29,30,31,32). CD8+ cytotoxic T lymphocytes (CTL) especially have been demonstrated to recognize and kill cancer cells in various tumor models (33,34,35). As shown in a murine bone marrow-derived DC model, the successful activation and proliferation of T cells may also be
dependent on B7/CD28 costimulation between the DC and T cell -- with the appropriate and timely presence of T helper 1-associated cytokines (such as IL-12, tumor necrosis factor alpha, IFN-gamma). Depletion of either CD4+ or CD8+ T cells from tumor-bearing mice before therapy totally suppresses the therapeutic efficacy of DC pulsed with tumor-derived peptides (29). This suggests the need for both the CD4+ and CD8+ T cell populations for effective cancer immunotherapy.

Most of the ‘therapeutic’ successes in mice have been accomplished using antigenic tumor peptides for pulsing of DC (29, 31, 32, 33, 34, 35). Endogenous complex tumor antigens can be processed and presented via the proteasome into the major histocompatibility (MHC) class I compartment onto the DC cell surface within the context of MHC class I molecules, or alternatively peptides can bind directly onto the DC cell surface class I molecules if they have the correct binding motif (36). However, perhaps for more long lasting, sustained and effective therapeutic vaccination in cancer immunotherapy, T-helper cell (CD4+) stimulation and activation should also occur for sustainable CD8+ T lymphocyte activation and proliferation. The T-helper cells provide cytokines such as IFN-gamma and IL-2, which help activate the CD8+ population. DCs can also process and present exogenous antigens via lysosomes into the MHC class II compartment (36). MHC class II presentation of exogenous antigen (with concomitant costimulation of CD80 and CD86 and CD40 ligand binding) by antigen presenting cells such as DCs activates the CD4+ helper population. Moreover, long term survival of mature memory CD4 T lymphocytes has been shown to be dependent upon major histocompatibility class II-expressing dendritic cells (37).

In addition, DCs also have alternative pathways of antigen processing and can route exogenous antigen taken in by macropinocytosis into the MHC class I pathway through a mechanism known as cross-priming (38). Macropinocytosis is dependent on membrane ruffling, which is inhibited by amiloride. Amiloride pretreatment before exposure of DCs to soluble ovalbumin ultimately blocks presentation of an H-2K^b- restricted ovalbumin 257-264- peptide to B3 (SIINFEKL) CTL clones as measured in a Cr^51 release assay (21). In the endoplasmic reticulum (ER), peptides from cytosolic antigens bind to newly synthesized MHC class I molecules. Brefeldin A inhibits vesicular movement from the ER and Golgi complex and thus precludes presentation of peptide-MHC class I complexes. Brefeldin A treatment inhibits DCs ability to present ova (via exogenously added ovalbumin) but not presentation of exogenously added synthetic
ovalbumin peptide 257-264 (SIINFEKL) (21). This provides more evidence that peptides processed from soluble ovalbumin, an exogenous antigen, are loaded on MHC class I molecules in the ER.

Thus, exogenous proteins (e.g. from tumor cell lysates) can provide a larger repertoire of potential antigens, including neo-antigens, than for example, singular synthetic peptides that can be processed and presented by DCs, and (upon co-stimulation) prime either or both the CD4+ and CD8+ T cell populations to cause functional killing of tumors. Nonetheless there is no clear evidence that DCs capture and process antigens from malignant cells in-vivo. Additionally, tumors can produce immunosuppressive factors such as IL-10 (39,40) and vascular endothelial factor (41) that can subsequently decrease DC development and function. Although cytotoxic T lymphocytes may recognize MHC class I-peptide complexes on the tumor cell surface (e.g. melanoma), without DC-mediated helper T cell function CTL precursors are not expanded and CTL exhaustion can ensue. Therefore, tumors may have tumor specific or tumor associated antigens that T cells can recognize but they may not be immunogenic due to a lack of DC mediated APC function, which results in the immunosuppressive environment of the tumor bearing host.

**Dendritic cells in ex-vivo immunotherapy**

To address the effect on APC function mediated by the deficient immunogenic environment of the tumor milieu and harness the potency and specificity of the immune system, other ex-vivo procedures for APC (DC) activation may prove fruitful. For example, immature human DC provided by ex-vivo culture of peripheral blood monocytes can be pulsed with tumor antigens, exposed to the requisite maturation agents needed for upregulation of costimulatory molecule expression (for costimulation with T cells), and chemokine receptor expression (for migration to secondary lymph) and subsequently reintroduced back into the tumor bearing host.

*Ex-vivo* adoptive transfer of antigen-pulsed, mature autologous dendritic cells has been considered as a strategy for immunotherapy in humans. It has been shown that in mice 6 hours after intravenous injection of LPS there are increased numbers of labeled DC found in the T cell area of the spleen (42). These DC were found to also show increases in the B7 costimulator and T cell stimulatory capacity with a downregulation of processing capacity. Could the profound effects of LPS and LPS-like compounds be used to mature or develop antigen-pulsed DCs outside the living organism to ultimately promote a TH1 type of response in-vivo when reintroduced back into the body? In the mouse bone marrow there is an
enormous reservoir of monocyte DC precursors that can be utilized for this purpose. Numerous
investigators have demonstrated that murine bone marrow derived DCs are able to promote prophylactic
and therapeutic antitumor immunity when pulsed with relevant tumor-associated T cell epitopes
(29,30,32,34). Yet, antitumor immunity is dependent on CD4+ T cell help even when bone marrow
derived DCs are only pulsed with peptide to induce CTLs proliferation (43). In addition, T helper type 1
development of naïve CD4+ T cells requires the coordinate action of IL-12 and IFN-gamma (44). Lastly,
the capacity to induce an antitumor immune response in-vivo correlates to the degree of DC (i.e. bone
marrow derived) maturation.

Several methods exist to grow relatively large numbers of immature DC recovered from peripheral
blood (human) (45) or bone marrow (mouse) (5,46). In the mouse, tumor lysates or antigenic peptides
have been successfully used to ‘pulse’ or load immature syngeneic DC ex-vivo and subsequently
reintroduce back into the animal eliciting both protective tumor immunity or regression of existing tumor
(8,29). Mice injected with bone marrow derived DC prepulsed with a cytotoxic T lymphocyte-recognized
tumor peptide, develop specific cytotoxic T lymphocyte response and are protected against subsequent
tumor challenge with tumor cells expressing the relevant tumor antigen (47).

**Lipid A mimetics can serve as adjuvants for maturation of the DC**

LPS is known to promote severe inflammation leading to tissue destruction (48). It can also activate
macrophages, which are derived from the same myeloid lineage as the dendritic cells, to produce large
amounts of pro-inflammatory cytokines such as TNF-alpha, IL-1-beta and IL-6 that attract and stimulate
other inflammatory cell types including T cells (49,50). Besides cytokines, nitric oxide and eicosanoids
released by macrophages can be inducers of LPS-induced septic shock caused by Gram-negative infection.

Even washing away as much LPS as possible after exposing DCs ex-vivo to this maturation agent, it is
perhaps too toxic a regimen for use even as an ex-vivo maturational agent for human DC after they have
taken up antigen. Corixa’s (formerly Ribi ImmunoChem Research, Inc.) adjuvants, which are attenuated
derivatized versions of LPS, including MPL-S® (51) and selected, synthetic mimetic compounds, may
provide a wider and effectively safe dose range as an adjuvant for a number of human vaccines. Another
use for these adjuvants may be the ex-vivo maturation of cultured, antigen pulsed DC for use in
immunotherapeutic approaches.
LPS has been shown to be a "potent adjuvant for protein antigens, and, furthermore possesses the unusual property of acting as an adjuvant even if administered at a different site and at a different time than the antigen (52, 53, 54). "This property set LPS apart from other adjuvants in use at that time, such as alum or complete Freund's adjuvant, since these materials had to be directly associated with the antigen in order to be effective." Ribi et al. found that reduction of the endotoxic activities of LPS without loss of its ability to regress tumors could be achieved by exposure of LPS to mild acid hydrolytic conditions yielding monophosphoryl lipid A (MLA) with only a single phosphate at the 4' position (the nonreducing end) of what is just a diglucosamine moiety as a result of the loss of also the inner core residues attached via the 6 position (55). Subsequent work found that additional treatment with mild alkaline specifically removed one fatty acid from monophosphoryl lipid A at the 3 position leaving a free hydroxyl group, which produced more attenuation of its toxicity but no change in immunostimulating activity (56) (see Figure 1). These chemical modifications of LPS led to the creation of a new product called MPL-S® which is actually a combination of 3-O-deacyl-4'-monophosphoryl lipid A species having basically two attributes. One is the inherent variable incorporation of ester-linked normal fatty acids in the lipid A structure during biosynthesis. The other cause of heterogeneity in MPL-S® is the variable loss of ester-linked fatty acids from the 'incomplete chemoselectivity' during the hydrolytic steps (especially the acidic step) in the production process, which can yield 'several less highly acylated compounds in addition to hexaacyl component' (54, 57). This heterogeneity in structure manifests the cumulative contributions of each of its constituent species. Most importantly, perhaps, for maximal immunostimulant activity the series of 3-O-deacyl-4'-monophosphoryl lipid A should be hexaacylated with a free hydroxyl group at the 3 position of the diglucosamine backbone (57). This observation is similar to other studies (58) showing that a β-(1 to 6) diglucosamine moiety bearing six pendant fatty acids is a prerequisite to the full expression of endotoxic activities (57). A schematic representation of the production process for MPL-S® is shown in Figure 1. LPS R595 from Salmonella minnesota R595 is the parent compound of MPL-S® and these are both tested in the proceeding thesis project.

In addition, several synthetic compounds that mimic MPL-S® in structure and function exist and are compared in this thesis as well. As pictured in Figure 2 they are similar to MPL-S® in that all of them have 6 ester-linked fatty acid acyl chains and a sole phosphate group in the non-reducing 4 position (if having a
monoglucosamine moiety) or 4’ position (if a diglucosamine moiety). Thus, the synthetic lipid A mimetics studied here differ in terms of having a glucosamine mono or disaccharide moiety, the length of the 6 ester-linked fatty acid acyl chains and one other aspect. For the lipid A mimetics having a glucosamine monosaccharide moiety (RC527, RC529 and RC544) there is a substitution on the second carbon, which is an amide linkage with the di-acyl chain, and which helps form the acetal linkage to carbon number one of the glucosamine monosaccharide of the lipid A mimetics. RC529 is similar in structure to RC527, but RC529 has all chain lengths of 14 and has only a single hydrogen substitution where RC527 instead has the carboxyl group substitution on the second carbon just described above. Moreover, RC544 is similar to RC527 except that the chiral carbon has the amide linkage for the acyl chain stereospecifically different and has a hydroxyl substitution where the carboxyl group is in RC527. RC511, the only lipid A mimic studied here having a diglucosamine moiety, and RC527 both have acyl carbon chain lengths of 10 and 14. Furthermore, RC529 has all acyl chain lengths of 14 and RC544 has 3 diacyl pairs in a combination of 12 and 14 chain lengths.

Thus, the structural progeny of LPS R595, including MPL-S® and the synthetic lipid A mimetics RC511, RC527, RC529 and RC544, were chosen as candidates for maturation of murine, bone marrow derived DCs for the following reasons. Previous animal and human clinical studies using MPL-S® have met good safety and efficacy criteria and commercial potential. Previous biological screenings measuring the direct effects of a wide range of synthetic lipid A mimetics on the human monocytic leukemia cell line THP-1, to secrete the proinflammatory cytokines including TNF-alpha, IL-1 beta, IL-8 and IL-6, made RC529 and RC544 early candidates for further development. Likewise, earlier developmental work has shown that both RC527 and RC511 are potent biological adjuvants (i.e. pyrogenic) even at very low doses compared to MPL-S® and RC529 and RC544. It may be that a pro-inflammatory milieu is important for obtaining effective antigen loaded DCs to mature into the most potent antigen presenting cells possible.
Figure 1. A schematic representation of the production process for MPL®. The structures shown are the most highly substituted forms that are observed at each step. Dashed lines are used for bonds to groups that occur in nonstoichiometric amounts. The structure for MPL® corresponds to that shown for 3D-MLA. Reprinted from (54).
Figure 2. Structures of synthetic lipid A mimetic compounds RC511, RC527, RC529 and RC544.
Research Objectives

This thesis focuses on the maturation effects of lipid A mimetics on the, in-vitro, antigen presenting capabilities of murine, bone marrow-derived dendritic cells in an antigen specific T cell effector model. Specifically studied is the upregulation of maturational and functional cell surface marker expression on murine dendritic cells following antigen pulsing and lipid A mimetic stimulation. The secretion of known proliferative cytokines such as IL-2 and IFN-gamma indicative of T cell activation subsequent to cognate accessory cell interaction with the antigen specific CD4+ effector T cell population can be increased through maturation of the DC by the lipid A mimetics.

**Major Objective:** The major objective of this study was to characterize the influence of selected lipid A mimetics on the maturation and functional development of murine bone marrow derived dendritic cells and evaluate their effectiveness as antigen presenting cells after lipid A mimetic stimulation in-vitro.

**Specific Aims:**

1. Maturation of bone marrow-derived murine dendritic cells by lipid A mimetic compounds were to be compared using flow cytometry through the measurement of the cell surface expression of the co-stimulatory cell surface markers, CD80 (B7.1), CD86 (B7.2), CD40 receptor and the major histocompatibility (MHC) class II restricted molecule IA^d_. These are cell surface markers believed to be characteristic of mature antigen presenting cells (DCs). Dendritic cells that have taken up antigen are induced to express those maturation/activation markers.

2. Determine the maturational and functional effects lipid A mimetics have on the antigen presenting capabilities of bone marrow-derived, murine DCs. The project work determined if bone marrow-derived dendritic cells that have processed exogenous protein antigen (ovalbumin) and have subsequently been induced to mature by various lipid A mimetic compounds are effective antigen presenting cells when put in the presence of naïve T cells as measured by the amounts of interferon-gamma and interleukin 2, cytokines produced by activated T cells. The syngeneic DO11.10 mouse model was chosen for this purpose. CD4+ T cells from transgenic DO11.10 mice, can be quantified
by flow cytometry using a monoclonal antibody (KJ1) specific for the transgenic ova peptide specific TCR complex.

3. Quantify the secreted amounts of IL-2 and IFN-gamma as indices of T-cell activation in the DO11.10 functional assay. The enzyme-linked immunosorbant assay (ELISA) was chosen for this purpose. Also, measure by ELISA the DC derived cytokines IL-12 p40 and bioactive IL-12 p70, which are believed to be important for helping drive a T helper cell type 1 (Th1) immune response.
CHAPTER 2

Materials and Methods

Amphiphiles, reagents and growth factors. All LPS (from *S. minnesota* R595), from which MPL-S® is derived, and synthetic lipid A preparations were provided by Corixa (Seattle, WA.), previously created at Ribi ImmunoChem Research, Inc. (Hamilton, MT). Stock solutions of amphiphiles were at 100 ug/ml in 10% ethanol and stored at 4°C. Tissue culture grade 1X PBS, chick ovalbumin grade VII, essentially free of S-ovalbumin (Sigma cat. no. A 7641), cell culture and endotoxin tested penicillin-streptomycin solution containing 10,000 U penicillin and 10 mg per ml in 0.9% NaCl (cat. no. P 0781) and 2-mercaptoethanol (cat. no. M 7522) were obtained from Sigma (St. Louis MO). Working stocks of ovalbumin at 1.0 mg/ml were stored at −70°C until used. Recombinant murine GM-CSF and human natural TGFβ1 growth factors were obtained from Peprotech (Rocky Hill. NJ). Working stocks of rmGM-CSF were made at 100ng/ul and stored at −20°C while the human TGFβ1 was made at 100ng/ul and stored at 4°C until used. Endotoxin levels for the growth factors were found to be below 0.1 ng per µg (1 EU/µg) of GM-CSF or TGFβ1. Media solutions and supplements also had endotoxin levels found to be less than the detectable levels by the Limulus assay.

Bone Marrow Acquisition. Fresh bone marrow growth medium was made with RPMI 1640 (Mediatech cat. no. 10-040-CM from Fisher Scientific) supplemented with 10% heat inactivated fetal bovine serum (HyClone Laboratories, Logan UT). 2 x 10^-5 M 2-mercaptoethanol, and 100U/ml penicillin plus 0.1 mg/ml streptomycin. Bone marrow cells were obtained from femurs of 6 to 12-week-old female Balb/c mice (Jackson Laboratory, Bar Harbor, ME). Prior to excision from the mouse the femurs were aseptically cleaned of connective tissue. A sterile 22 gauge needle and syringe filled with fresh RPMI containing 10% FBS was used to flush out the ‘plug’ of bone marrow cells. After vigorous pipetting the cell suspension was allowed to stand for approximately one minute. Any debris, which had settled after a brief standing, was removed aseptically. Between 8 to 15 million cells per mouse were acquired in this fashion. Immediately before final resuspension and distribution of cells into petri dishes for initiation of 10 day cultures 20 ng/ml of recombinant GM-CSF and 50 ng/ml TGFβ1 were added to the bone marrow growth media.
Cell Culture. The culture conditions and feeding schedule essentially followed the method of Lutz et al. (46) with the supplemental addition of human, natural growth factor TGFβ1 at 50 ng/ml. TGFβ1, a known potent immunosuppressive cytokine, was utilized since it was found to yield immature DCs (collected predominantly from the nonadherent population of murine bone marrow cells cultured up to 8 days in the presence of GM-CSF and TGFβ1) but only when also in the presence of Fc-bearing macrophages left in the same culture. If, however, only the FcR+ depleted populations were allowed to grow for the last two days of culture (Days 6 to 8) in the presence of only GM-CSF (i.e. with no TGFβ1) there was a very distinct upregulation of B7.2 (CD86) on the high MHC class II population commonly considered to be DCs. In fact, this shift in the number of more mature cells is seen in the first 6 days of culture whether FcR+ depleted populations or not are cultured in the GM-CSF alone in contrast to culturing with both GM-CSF and TGFβ1 (59). Applying the tissue culture technique of Lutz et al. the bone marrow-derived cells were fed at Days 0, 3, 6 and 8 with harvesting of nonadherent population of cells on Day 10 described as follows. At Day 0 the aseptically obtained bone marrow cells were seeded at 2 x 10^5 cells/ml in 10ml of growth media using 100 x15mm microbiological grade dishes (Fisher Scientific). At Day 3 an additional 10ml of fresh growth medium also containing fresh 20 ng/ml rmGM-CSF and 50 ng/ml human, natural TGFβ1 was added to existing media and cells. At Days 6 and 8 a volume of 10ml of media also containing some cells was aseptically pipetted from each petri dish and centrifuged at 300g for 8 minutes. Cells were resuspended in 10ml fresh media containing fresh growth factors. At Day 10 all non-adherent and any loosely adherent cells were harvested by vigorous pipetting with the existing media and then washing the plate again with vigorous pipetting with cold 1X PBS supplemented with 1% FBS. The consolidated cells were then washed one more time before being ready for antigen pulsing and lipid A inductions.

Establishment of homozygous DO11.10, KJ1+ transgenic mice colony. Homozygous breeding pairs were obtained from Dr. P. Marrack (National Jewish Hospital- Denver). Mice were maintained with brother-sister breeding pairs at Ribi ImmunoChem Research Inc.

Negative Selection of Naïve CD3+ T-cells. Enrichment for naïve CD3+ T cells occurred as follows. Splenocytes were aseptically obtained from spleens of homozygous, KJ1+ DO11.10 transgenic mice from the same sex that which the bone marrow-derived dendritic cells were obtained from the background Balb/c strain. Gentle scraping of spleens in 1X PBS containing 1% FBS, vortexing cell mixture and allowing the
settling of tissue debris yielded between 50 to 150 x 10^6 cells/spleen. Not more than 200 x 10^6 splenocytes as counted by trypan exclusion were loaded per CD3+ T-cell column (R&D Systems cat. no. MTCC). B cells and monocytes were positively selected by antibodies attached to the column allowing CD3+ T cells to pass through and be collected. Both pre- and post -column cells were stained with biotinylated KJ1 antibody and strepavidin APC and anti- CD4 FITC antibody. Following manufacturers instructions post-column cell counts varied between 7% and 18% when comparing to pre- column cell counts. Cell enrichment for CD4+, KJ1+ identity increased from approximately 18 % to 56% between pre- and post column cell populations.

Preparations for Flow Cytometry Analysis and the DO11.10 Functional Assay:

Ovalbumin-antigen pulsing. All the harvested and washed non-adherent cells were resuspended in fresh bone marrow medium containing only 10 ng/ml rmGM-CSF at 1 x 10^6 cells/ml. TGFβ1 was not added back to these cultures since it has been shown to inhibit the synergistic action of IL-12 and IFN-gamma in the T helper cell development of naïve CD4+ T cells (60). As an untreated control 5ml of these cells were re-plated in a 60 x 15mm microbiological grade petri dish (Fisher Scientific). The remaining cells received the 10ug/ml chick ovalbumin (Sigma grade VII) dose for pulsing and were also immediately re- plated in 5ml volumes at 1 x 10^6 cells/ml in 60 x 15mm microbiological grade petri dishes. Antigen pulsing was done at 37° C in the presence of 5% CO2 for 4.5 to 5 hours before subsequent lipid A inductions. At least one dish was maintained as the ovalbumin –antigen only pulsed control.

Lipid A inductions. Working stocks of each respective lipid A were made fresh immediately before administration to individual plates. No more than 125ul was added to the 5ml of cells for any of the doses administered. After 18-24 hours of treatment in the presence of the lipid A mimetic all cells were removed including the apparently adherent as well as the non-adherent cells by the following method. Each petri dish had any non-adherent or loosely adherent cells removed by vigorously pipetting. Five ml of cold 1X PBS containing 1% FBS was then added to the petri dish. Next, a sterile rubber policeman was used to gently loosen the adherent layer of cells from the surface of the dish. These cells were collected and an additional 5ml of cold 1X PBS was added immediately afterwards to help retrieve any remaining cells. These three volumes were consolidated. This harvest procedure was performed for each treatment group. Respective cell groups were washed 2X in cold 1X PBS containing 1% FBS before cell counting by trypan
blue exclusion. The cells were then ready for use in flow cytometry analysis or application in the DO11.10 functional assay as described later.

**Flow Cytometry Analysis.** After a total induction period of 24 hours which comprised an initial 4.5 to 5 hour ovalbumin pulse followed by a subsequent 18 to 24 hour lipid A induction with no washing of antigen from the culture system, cells were harvested as described above. The ovalbumin-pulsed, lipid A-induced, 10 day old dendritic cells were stained with FITC, PE (phycoerythrin) or biotin-conjugated monoclonal antibodies from PharMingen. They included:

1. I-A$^d$/I-E$^d$ (2G9) PE
2. CD11b (Integrin $\alpha_M$ chain, Mac-1 $\alpha$-chain) (M1/70) FITC
3. CD3-e FITC
4. CD19 (1D3) FITC
5. CD86 (B7-2) (GL1)
6. CD40 (3/23) PE
7. Ly-6G (Gr-1) FITC
8. GR-1 Biotin
9. CD80 (B7-1) Biotin
10. I-A$^d$/I-E$^d$ Biotin
11. CD11b (Mac-1 $\alpha_M$-chain) Biotin
12. Streptavidin-APC
13. Fc block- anti-CD16/CD32 (Fc$\gamma$II R) (2.4G2)
14. PE-rat IgG2a isotype control

Flow cytometry immunophenotyping of cultured murine bone marrow-derived dendritic cells was carried out using the following procedure:

1. DCs were added to 12x75 polystyrene staining tubes at 0.5 x $10^6$ cells in a final volume of 100ul of cold PBS containing 0.05% sodium azide and 2.0% BSA (standard ‘wash buffer’). Between 0.5 and 1.0ug/tube of pre-titered, fluorochrome-conjugated monoclonal rat or hamster anti-mouse antibody was added to each tube and gently mixed. All tubes received Fc blocking antibody to minimize non-specific binding. Cells were stained for 30 minutes at 4°C. Subsequently, sample tubes were washed with 3 mls of cold wash buffer and spun at 1300 RPM (300G) for 5 minutes, decanted and the cell pellets resuspended and washed a second time. After the second wash cells were resuspended in 0.5mls of a 1% buffered (pH 7.5) paraformaldehyde and stored at 4°C prior to analysis.

2. Isotype control samples consisted of cells from each DC induction test group that were stained with a cocktail of CD11b (Fitc)/$\gamma$1(PE conjugated)/streptavidin-APC. The concentration of the $\gamma$1 myeloma
control protein was 0.5-1.0 μg/tube (comparable to the staining test Mabs) and the F/P ratios for the isotype control reagents was equivalent to those for the test Mabs. Strepavidin-APC was added at the same concentration (1 μg/tube) used in the test stains.

3. For this study the most important antibody combinations for analysis were: the three antibody combination CD11b (FITC)/CD86 (PE)/CD80 (APC) and CD11b (FITC)/CD40 (PE)/I-A^d (APC). These two three-color mAb combination stains allowed the enumeration of cultured cells that were CD11b positive and simultaneously positive for I-A^d and CD40 or simultaneously positive for CD80 and CD86. All immature DCs are moderately I-A^d positive but weakly positive for the T cell co-stimulatory molecules CD40, CD80 and CD86. Maturation induction by an activation agent such as TNF-alpha or LPS drives the increase of high levels of CD40, CD86, CD80, and MHC class II antigen expression on the cell surface of dendritic cells.

4. All samples were analyzed on a Coulter Elite, three laser flow cytometer. FITC and PE fluorescence was excited with a 488 nm emission wavelength, argon laser, and APC was excited using a 630 nm emission wavelength, helium-neon laser. Isotype controls for each DC test group were first analyzed to establish boundary cursors to delineate positive and negative staining regions for each antibody. Data were analyzed by first gating on the 90° versus forward angle light scatter array of the cultured DCs. 10 day cultured DCs were > 90% pure by light scatter criteria. Subsequently, the various three-color (three antibody) stains were analyzed using a series of two parameter histograms as shown in Figures 2 and 3. Ten fluorescent event histograms were collected for each sample analyzed.

**Antigen presenting cell and T cell functional assay: The DO11.10 functional assay:**

Accessory cell function and T-cell activation were measured in the DO11.10 functional assay. Briefly, after washing and counting by trypan blue exclusion individual pulsed and lipid A-induced dendritic cell treatment groups were titrated by 1:2 dilutions across U-bottom 96 well polystyrene plates (Costar cat. no. 3799) in fresh bone marrow medium devoid of growth factors. Mature dendritic cells were first resuspended at 1 x 10^6 cells/ml with 200μl administered to the first well. Second and subsequent wells already containing 100μl each of fresh media were used to titer 100μl aliquots of the respective dendritic cell population in which the last column of cells had 100μl discarded. Starting at column three and working across 150μl containing 2.5 x 10^5 of negatively selected naïve, CD3+ T-cells were added on top of the
dendritic cells yielding a starting ratio of 5:1 with subsequent ratios of 10:1, 20:1, 40:1 and 80:1. This produced 250ul/well and consecutive dendritic cell: T-cell ratios differing by a factor of 2. One row of ovalbumin only pulsed controls were also set up in the presence of naïve, CD3+ T cells. Dendritic cell only control groups were also done representing each of the respective induction treatments, in which 100ul of bone cell media was added instead of naïve T cells. Lastly, a no treatment control was run which had dendritic cells, that were neither pulsed nor induced by lipid A (This was primarily for measurement of constitutively expressed IL-12 p40 by dendritic cells.). Plates were briefly centrifuged at for 2 minutes at 50g to permit potentially closer contact between the APC and T-cells. At 24 hours a 30ul aliquot was aseptically taken, diluted in 120ul of media, and stored at -70°C until IL-2 measurement. At 44-48 hours three more 30ul aliquots were taken and also diluted in 120ul of media for IFN-gamma and IL-12 p40 measurement. In addition, a 50ul aliquot was taken and diluted in 100ul of media dispensed on polypropylene plates for IL-12 p70 measurement. All of these aliquots were stored at -70°C until ELISAs were performed.

**Cytokine elaboration.** Quantikine ELISA kits for IL-2, IFN-gamma, IL-12 p40 and IL-12 p70 were obtained from R&D Systems (Minneapolis, MN). As practical as possible dilutions of samples, in particular for IFN-gamma and IL-12 p40 measurement, were made such that observed O.D. values fell above the lowest standard and yet below the second highest standard. Generally, no further dilutions had to be made for measurement of IL-2 and IL-12 p70.
Chapter 3

Results

Flow Cytometry Analysis Characterizing the Maturational Development of Murine Dendritic Cells through Induction by Lipid A Mimetics

To compare the efficacy of lipid A mimetics in enhancing the antigen presenting capabilities of DC's the DO11.10 T Cell Receptor (TCR) transgenic mouse model was employed. The germline DNA of DO11.10+ TCR-transgenic mice contain rearranged T cell receptor (TCR)-V alpha and V-Beta genes that encode a TCR specific for chicken ovalbumin peptide 323-329 bound to I-A<sup>d</sup> class II MHC molecules (61). Although not performed for this thesis project, adoptive transfer of ex-vivo antigen-pulsed, lipid A-induced DC's can be performed to study immunotherapeutic approaches. Using flow cytometry this transgenic TCR can be detected with the KJI-26 monoclonal antibody that binds only to this particular TCR heterodimer (62). Thus, the number of antigen-specific MHC II restricted CD4+ T cells can be quantified by flow cytometry. In addition, the DO11.10 mice are syngeneic to the Balb/c since it has been backcrossed more than 10 times to the Balb/c background. This allows for the study of natural, exogenous whole protein displayed as processed antigen in a MHC restricted fashion on bone marrow derived (Balb/c), syngeneic DCs for presentation to the transgenic T cells. Hence, barring any MHC I restricted presentation of other ovalbumin antigenic determinants, cytokines secreted in the DC/T cell functional assay are not the result of nonspecific stimulation typically found in allogeneic mixed lymphocyte reactions. Instead, the level of cytokines secreted are the result of predominantly syngeneic, MHC class II restricted antigen specific presentation by DCs to naïve T cells. Specifically, can lipid A mimetic compounds trigger the maturation of the DCs in such a way that maximizes the antigen-presenting capabilities of initially ‘immature’ DCs which have processed or are actively processing ovalbumin? The maturation process induced by lipid A mimetic compounds would increase the cell surface expression of co-stimulatory molecules CD40, CD80 and CD86 and the concomitant display of I-A<sup>d</sup> MHC class II antigen-expressing molecules all of which are important in effective antigen-presentation and can be measured by flow cytometry.

The objectives in this chapter include demonstrating by flow cytometry analysis the maturation of DCs by the inductive effects of MPL, R595 LPS and selected lipid A mimetics. In addition, a second goal the number of KJ1+, MHC II, ovalbumin-specific naïve T cells enriched by negative selection from
DO11.10 splenocytes are quantified. Once quantified they are used in specific ratios in the T cell functional assay with DCs which have been pulsed with whole protein antigen, and then either matured in the presence of lipid A mimetics or not. Proliferative and stimulatory cytokines IL-2 and IFN-gamma and the DC derived IL-12 p40 and bioactive IL-12 p70 are measured by ELISA.

**Results.** The top row of dot plots in Figure 1 show that the dot plots for the isotype controls (negative control stains) for the gated dendritic cells displayed in the two parameter forward angle versus 90° light scatter histogram. As shown, both the untreated DCs and DCs treated with MPL, while positive for CD11b (FITC) expression, are negative for any nonspecific staining with the PE and APC labeled isotype control fluorescent stains. Figure 2 shows that treatment of antigen pulsed DC’s with R595 LPS, MPL-S and various lipid A mimetics caused significant upregulation of the costimulatory molecules B7.1 (CD80) and B7.2 (CD86). Specifically, only 29.9% of the nontreated (no antigen pulse and not lipid A induced) control DC population (upper right quadrant of the top histogram in the third column of histograms) concomitantly expressed high levels of both CD80 (B7.1) and CD86 (B7.2). By comparison all of the lipid A induced groups with the exception of RC529 showed an increase to over 85% and above for dual expression of CD80 and CD86. RC529 stimulation, however, yielded a modest 44.9% level of CD80/CD86 co-expression on the antigen pulsed, lipid A induced DCs which albeit was still higher than the no treatment control.

In Table 1 analysis of dual stained CD80+ and CD86+ cells reveals that this pattern of induction is also reflected for the respective treatment groups when the mean channel fluorescence (MCF) quantitation is determined. MCF is a measure of the average number or density of the cell differentiation markers on each cell. For CD86 expression, R595 LPS, MPL-S and all of the lipid A treated groups, except RC529, demonstrated increases yielding 40 to 49 channels (4 decade log scale) above the no treatment control. By contrast, RC529 stimulated only 19 channel increase over no antigen pulse, no lipid A induced dendritic cell control levels. Similarly the CD80 increase was even greater, showing greater than a 3 fold increase in MCF for all lipid A mimetics except RC529, which stimulated only a 20 channel increase in CD86 cell surface fluorescence over the background control. This indicates that the predominant effect of all the lipid A treatments, except RC529 which showed an overall weaker effect, is that a much greater number of murine bone marrow-derived dendritic cells become positive for both co-stimulatory molecules CD80 and
CD86 after *in-vitro* stimulation compared to the no antigen and no lipid A induced treatment control dendritic cells.

**ISOTYPE CONTROLS**

![Flow Cytometry Isotype Controls](image)

**Figure 1.** Flow cytometry isotype controls from one representative experiment showing the no treatment and lipid A treatment groups. Results are from one representative experiment. Isotype control samples consisted of cells from each DC induction test group that were stained with a cocktail of CD11b (Fitc)/γ1(PE conjugated)/streptavidin-APC. The concentration of the γ1myeloma control protein was at 0.5-1.0ug/tube (comparable to the staining test mAbs) and the F/P ratios for the isotype control reagents was equivalent to those for the test mAbs. Streptavidin-APC was added at the same concentration (1ug/tube) used in the test stains.
Figure 2. Comparison of the inductive effects of MPL, R595, LPS and selected lipid A mimetics for the cell surface upregulation of CD80 and CD86 expression on immature CD11b positive murine bone marrow-derived dendritic cells. Flow cytometry analysis of immature DCs stimulated for 24 hours with the indicated molecular species and subsequently stained with the three antibody combination CD11b(Fitc)/CD86(PE)/CD80(APC). Two parameter histograms in the first and second columns show the concomitant staining patterns for CD11b(Fitc)-x axis and CD86(PE)-y axis, or CD11b(Fitc)-x axis and CD80(APC)-y axis respectively. Figure 2 legend continued on next page.
Figure 2 legend continued. The two parameter histograms in the third column of Figure 2 show the level of concomitant staining for both CD86 and CD80 on CD11b positive, cultured DCs. The percentage of dual positive DCs for each induction condition are indicated in the upper right hand quadrant of each histogram in the third column of Figure 2.

Table 1. Mean channel fluorescence (MCF) of co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) staining dual positive with stimulated CD11b+ murine bone marrow-derived DCs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD86</th>
<th>CD80</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>95</td>
<td>25.8</td>
</tr>
<tr>
<td>LPS 10 ng/ml</td>
<td>143</td>
<td>114</td>
</tr>
<tr>
<td>MPL-S 100 ng/ml</td>
<td>135</td>
<td>101</td>
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<tr>
<td>RC511 100 ng/ml</td>
<td>144</td>
<td>108</td>
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<td>RC527 100 ng/ml</td>
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<td>RC544 100 ng/ml</td>
<td>138</td>
<td>97.6</td>
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</tbody>
</table>
Table 2 and Figure 3 demonstrate two more important observations concerning lipid A induction of MHC class II antigens and CD40 cell surface expression on DCs. One observation is that lipid A inductions produce very little change in the proportion of gated cells which are constitutively dual positive for CD11b (FITC) and I-A<sup>d</sup> (PE) (85.2% for the no antigen, no lipid A treatment control, results not shown). Yet, there is greater than a two-fold increase in the density of I-A<sup>d</sup> cell surface expression as measured by mean channel fluorescence (MCF) after in-vitro stimulation with R595 LPS, MPL-S and the various lipid A mimetics (Table 2). RC529 yielded less of an effect than the other lipid A mimetics with a MCF = 1343 versus a range of the MCF=1677-2000 for the other lipid A mimetics but this was still more than the no treatment control = 787. A second, very important observation pertaining to lipid A stimulation is the dramatic increase in the percentage of DCs which become positive for both the CD40 antigen and class II antigen cell surface expression after in-vitro stimulation with LPS, MPL or the various lipid A mimetics. As shown in Figure 3 and Table 2 the no treatment (no antigen pulse, no lipid A induced) control group has only 12.4% of the total gated cell population being dual positive for CD40 and I-A<sup>d</sup> while this increases to between 87 to 93 % for LPS, MPL and all lipid A mimetic test groups, except RC529 which showed only 56% dual positive staining. A very similar pattern of percent positive was seen for CD40/CD11b co-expression in which the no treatment (no antigen pulse, no lipid A induced) control yielded 12.7%, RC529 reached 61.0%, but the R595 LPS, MPL-S and the other lipid A mimetics yielded over 90%, respectively. CD40 receptor interaction with the T-cell secreted CD40 ligand is considered important for IL-12 induction in DC and in mounting a T-helper cell type 1 mediated response (45).
Figure 3. Comparison of the inductive effects of MPL, R595, LPS and selected lipid A mimetics for the cell surface upregulation of MHC class II (I-A^d) antigen and CD40 expression on immature CD11b positive, murine bone marrow-derived dendritic cells. See legend description on the next page.
Figure 3 legend continued. Figure 3 shows the flow cytometry analysis of immature DCs stimulated for 24 hours with the indicated molecules species and subsequently stained with the three antibody combination, CD11b(Fitc)/CD40(PE)/I-A\(^d\)(APC). Two parameter histograms in the first and second columns show the concomitant staining patterns for CD11b(Fitc)-x axis and CD40(PE)-y axis, and for CD11b(Fitc)-x axis and I-A\(^d\)(APC)-y axis respectively. The two parameter histograms in the third column show the level of concomitant staining for both CD40 and I-A\(^d\) on CD11b positive, cultured DCs. The percentage of dual positive cells for each induction condition are indicated in the upper right hand quadrant of each histogram in the third column.

Table 2. Mean channel fluorescence (MCF) of CD40 and MHC class II (I-A\(^d\)) staining dual positive with CD11b+ murine bone marrow-derived DCs.

<table>
<thead>
<tr>
<th></th>
<th>I-A(^d)</th>
<th>CD40</th>
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</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>787</td>
<td>91.3</td>
</tr>
<tr>
<td>LPS 10 ng/ml</td>
<td>2000</td>
<td>128</td>
</tr>
<tr>
<td>MPL-S 100 ng/ml</td>
<td>1785</td>
<td>121</td>
</tr>
<tr>
<td>RC511 100 ng/ml</td>
<td>1801</td>
<td>129</td>
</tr>
<tr>
<td>RC527 100 ng/ml</td>
<td>1719</td>
<td>126</td>
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</tr>
<tr>
<td>RC544 100 ng/ml</td>
<td>1677</td>
<td>121</td>
</tr>
</tbody>
</table>
Compare the Efficacy of Various Lipid A Mimetics to Enhance the Maturational and Functional Development Dendritic Cells as Assessed by the Cytokine Elaboration in the DO11.10 in-vitro T-Cell Functional Assay

**Enrichment and identification of CD3+ T cells.** Staining and flow cytometry analysis of spleens cells before column separation for CD3+ T cells in Figure 4 shows that only 18.4% of the population of spleen cells are positive for both the CD4+, the T helper cell marker, and KJ1+ for the I-A\(^2\) class II, MHC restricted T cell receptor for ovalbumin. Figure 5 demonstrates that after CD3+ negative selection that 56.6% of the T cells were positive for the same antibodies and that the mean channel fluorescence, an indicator for cell surface marker density did not change as expected. The spleen cells were said to be enriched for CD3+ T cells of which CD4+ T cell helper population were a subset.
Figure 4. Demonstration by flow cytometry of KJ1+ staining of 'homozygous' DO11.10 mouse spleen cells before negative selection by passing over a CD3+ T cell separation column. Spleens cells were obtained, stained and analyzed as described in Materials and Methods.
Figure 5. Demonstration by flow cytometry of KJ1+ staining of negatively selected CD3+ T-cell from spleens of 'homozygous' DO11.10 mice. Cells were collected, stained and analyzed as described in Materials and Methods.

**IL-2 and IFN-gamma secretion.** Table 3 compares the ability of bone marrow-derived, antigen-pulsed DCs stimulated with the different lipid A mimetic compounds at the 100 ng/ml dose level to stimulate the secretion of IL-2 and IFN-gamma in the DO11.10 effector T cell assay. Clearly, as the T-cell: dendritic cell ratio increases the amount of respective cytokine diminishes. As expected the dendritic cells (DCs) that were not pulsed with ovalbumin but were put in the presence of DO11.10 T cells did not lead to the production of measurable amounts of either IL-2 or IFN-gamma, i.e. less than the sensitivities of the assays for both IFN-gamma (10pg/ml) and IL-2 (15 pg/ml). This suggests that although the culture media used throughout both the 10 day culture of the DCs and the T-cell: DC functional assay contained fetal bovine serum as a potential foreign antigen and even though it may have been processed and presented by the DCs in either a MHC class I or II fashion it did not activate the T cells. On the other hand, the ovalbumin only treated DC control does produce a fair amount of both IL-2 and IFN-gamma in the presence of ovalbumin specific DO11.10 T cells (having the TCR specific for chicken ovalbumin peptide 323-329 bound to I-A<sup>d</sup> class II MHC molecules (46) as might be expected when the whole foreign protein antigen is naturally processed and presented via MHC class II or even MHC class I by an ‘immature’ DC. Yet when the same population of ovalbumin treated DCs are also induced by a maturation agent the amounts of secreted IL-2 and IFN-gamma increase dramatically in the DO11.10 T-cell: DC stimulation assay. R595 LPS, MPL-S, RC511 and RC527 all induced comparable amounts of IFN-gamma. RC544 induced slightly less IL-2 than the other maturation agents at the 10:1, 20:1 and 40:1 T cell: DC ratios but at the 80:1 ratio RC544 induced an amount comparable to others. Moreover, at the 80:1 ratio MPL-S and all of the lipid A mimetics induce higher amounts of both IFN-gamma and IL-2 than the R595 LPS treatment group. RC529 apparently does not induce as much IL-2 or IFN-gamma as R595 LPS, MPL-S and all the other lipid A mimetics over the entire range of T-cell: DC ratios. Nonetheless, RC529 still produces more IL-2 and IFN-gamma than the ovalbumin only treated DC control. All the maturation agents, however, show induction of increased levels of functional secretion of cytokines over both control groups at all the T cell: DC ratios.
IL-12 p40 and IL-12 p70 secretion.

IL-12 p40 in combination with IL-12 p35 as a heterodimer constitutes the bioactive IL-12 p70. It is the bioactive IL-12 p70 which is known to help direct the development of TH1, cell-mediated type of response.

Table 4 demonstrates that for the entire range of T-cell: DC ratios all lipid A mimetic treatments led to profoundly greater amounts of IL-12 p40 than dendritic cell controls. The magnitude of the measured IL-12 p70 was different between experiments, and thus data are shown from one representative experiment in Table 4. The no treatment control and the antigen-only pulsed dendritic cell control cells demonstrated no measurable IL-12 p70 secretion. Conversely, both dendritic cell controls produced IL-12 p40 of almost equal magnitude at a T cell: DC ratio of 10:1. This may simply reflect that dendritic cells can constitutively produce IL-12 p40. The MPL-S and RC529 compounds led to no IL-12 p70 secretion. However, LPS R595, RC511, RC527 and RC544 all stimulated DC secretion of IL-12 p70 at the lower ratios (i.e. 5:1 and 10:1) of T cells: DCs. The evidence for the undetected IL-12 p70 secretion by dendritic cells treated with MPL-S is not conclusive, however, since in other experiments it has been shown to stimulate some (<100 pg/ml) secretion of IL-12 p70 (unpublished data).

Table 3. Representative experiment demonstrating the range of DC:T cell ratios used in the DOI11.10 functional assay. IL-2 and IFN-gamma secretion levels measured from supernatants harvested from in-vitro cultures of Balb/c bone marrow derived DCs and DOI11.10 spleen derived T lymphocytes. DCs were treated with ovalbumin antigen only or with ovalbumin plus various lipid A mimetics stimulation as indicated by the test groups shown in the first column of Table 3. Ratio values represent the T cell to DC ratios analyzed for each test group. Values are in pg/ml. Ten day old bone marrow derived dendritic cells were pulsed with ovalbumin for 4.5 hours then induced with respective lipid A molecules at 100 ng/ml (10 ng/ml for LPS R595) for 18-24 hours before being put together in in-vitro cultures in the presence of CD3+ T cells selected from ‘homozygous’ DOI11.10 mice as described in Materials and Methods. IL-2 and IFN-gamma measurements are from 24 and 48 hour supernatants, respectively. For amounts less than the sensitivity of the respective assay a “<” sign is indicated in front of the amount in pg/ml

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<th>Test groups</th>
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<th>IFN-gamma</th>
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<td>20:1</td>
</tr>
<tr>
<td>No treat.</td>
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<td>&lt;15</td>
</tr>
<tr>
<td>OVA only</td>
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<td>203</td>
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<tr>
<td>LPS R595</td>
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<tr>
<td>MPL-S</td>
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<tr>
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<td>811</td>
<td>372</td>
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<tr>
<td>RC544</td>
<td>1111</td>
<td>588</td>
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</table>
Table 4. Representative experiment demonstrating the range of DC:T cell ratios used in the DO11.10 functional assay. IL-12 p40 and IL-12 p70 secretion levels measured from supernatants harvested from in-vitro cultures of Balb/c bone marrow derived DCs and DO11.10 spleen derived T lymphocytes. Ratio values are the T cell to DC ratios studied in the in-vitro assay for each test group. Same experimental conditions as described for Table 1. ND= not determined. IL-12 p40 and IL-12 p70 measurements are from 48 hour supernatants. For amounts less than the sensitivity of the respective assay a “<” sign is indicated in front of the amount in pg/ml.

<table>
<thead>
<tr>
<th>Test group</th>
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<td>80:1</td>
<td>5:1</td>
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<td>20:1</td>
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<td>4348</td>
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<td>1282</td>
<td>793</td>
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<td>716</td>
<td>46</td>
<td>&lt;12.5</td>
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Figure 6. Direct measurement by ELISA of T-cell derived and DC derived cytokines secreted in the DC/T cell DO11.10 functional assay after stimulation of bone marrow derived dendritic cells with a single concentration of different lipid A mimetics. Ten day old bone marrow derived dendritic cells were pulsed with ovalbumin for 4.5 hours then induced with respective lipid A mimetics at 100 ng/ml (10 ng/ml for LPS R595) for 18-24 hours before being combined at a 10:1 ratio with T cells selected from homozygous DO11.10 mice as described in Materials and Methods. Values are averages of at least 3 (2 experiments for RC529) but not more than 5 experiments. IL-2 measurements are from 24 hour supernatants. IFN-gamma and IL-12 p40 measurements are from 48 hour supernatants. Error bars represent plus and minus one standard error of the mean. For IL-12 p40: A no treatment group was also included to show effects using dendritic cells which were neither antigen (Legend continued on next page.)
pulsed or lipid treated but were co-cultured with T-cells. Since IL-12 p40 is constitutively expressed by dendritic cells, and the DC secretion of IL-12 p40 increases after LPS stimulation, the IL12 p40 levels are thought to be DC derived in this experiment. The baseline ovalbumin pulsed, DC (with no lipid A treatment) control levels of cytokine levels for IL-12 p40, IFN-gamma and IL-2 are 101 +/- 34 pg/ml, 537 +/- 276 pg/ml, and 294 +/- 100 pg/ml respectively.

Measurement by ELISA of IL-2, IFN-gamma and IL-12 p40 produced in the DO11.10 functional assay.

Figure 6 reveals the average level of secretion from at least three experiments of IL-2 and IFN-gamma, cytokines associated with the proliferation and stimulation of T cells, in the DO11.10 functional assay. Clearly, for both IL-2 and IFN-gamma all the lipid A mimetic treatments of ovalbumin, pulsed DCs produced much greater amounts of cytokines than the ovalbumin-pulsed DC control. This was especially true for IFN-gamma as lipid A mimetic groups yielded thousands of pg/ml more than ovalbumin only pulsed control. Likewise, for IL-12 p40 it is clear that the lipid A treated DCs (except for RC529) had from over 1000 to 4000 pg/ml greater IL-12 p40 over the ovalbumin only treated DC control. In addition, and as mentioned in the interpretation of the representative set of data in Table 4, the ovalbumin only DC control showed no significant difference in secretion of IL-12 p40 than the no treatment control demonstrating that the induced secretion in this functional assay could be primarily an effect of the lipid A mimetic treatments of the DCs and not an effect of requisite antigen uptake and processing alone.
Figure 7. Normalized analysis of cytokines secretion levels measured in the DO11.10 functional assay after stimulation of bone marrow-derived DCs with 100 ng/ml of selected lipid A mimetics (10 ng/ml for LPS R595). All the data are from the same experiments used in Figure 6. Absolute values for cytokine levels induced by each lipid A test molecule are normalized to the effect of the antigen only control group in each experiment by taking the ratio of the lipid A induced cytokine values divided by the antigen-only control values in each separate experiment. All ovalbumin antigen only control groups are represented graphically at 100% -- data are displayed (y-axis) as a percentage of the ovalbumin antigen only induction effect. Error bars represent plus and minus one standard error of the mean.
Figure 7 represents the same data as contained in Figure 6 but the maturation-induced treatment groups are expressed as a percentage of ovalbumin pulsed only DC controls. There was inherent differences in the magnitude of secreted cytokines between Do11.10 functional assays and thus test groups from each individual experiment were compared to their respective controls to make better comparisons between experiments. The bar graphs and standard deviations shown in Figure 7 are averages of the experiments in which each respective ovalbumin pulsed only DC control group is compared to the respective lipid A mimetic groups within the same respective experiment. Clearly, all the lipid A mimetics, except RC529, yielded 2 fold to over 40 fold greater values in cytokines secreted over antigen only pulsed controls. For both IL-2 and IFN-gamma there appears to be no significant difference between the effects of the different lipid A mimetics, except RC529 which was much less than the other lipid A mimetics and not significantly different than ovalbumin only pulsed controls. For IL-12 p40, however, there does appear to be differences in lipid A treatments. LPS R595, RC511 and RC527 appear to yield similar levels of IL-12 p40 and be significantly higher than RC544, MPL-S® and RC529.
Figure 8. Dose response to lipid A mimetic in one experiment comparing MPL-S to RC527 and RC529.
Figure 9. Dose response to lipid A mimetic in one experiment comparing MPL-S to RC511 and RC544.
Figures 8 and 9 compare the elaboration of IL-2, IFN-gamma and IL-12 p40 from MPL-S® versus the other lipid A mimetics over a dose range (10, 50, 100, 250 and 500 ng/ml) from two different experiments. Please note that the magnitude of the cytokine secreted by MPL-S® between the two experiments is different yet the patterns of the respective lipid A mimetics are compared with respect to the MPL-S® when making this analysis. These dose response studies reveal that for both IL-2 and IFN-gamma at lipid A doses of 50 ng/ml and above, there is no significant difference between MPL-S® and lipid A mimetics RC527 and RC511. One exception is that at the 500 ng/ml dose the IFN-gamma level is two-fold greater for MPL-S® compared to RC511 treatment. In fact, the lowest dose tested of 10 ng/ml for RC511 and RC527 yielded similar IL-2 and IFN-gamma levels compared to all the other doses. In addition, although it might appear that RC527 does show an increasing dose range effect for IFN-gamma and IL-12 p40 secretions, the data still suggest that lower doses would have to be tested for both RC511 and RC527 to see a genuine dose range effect. RC544 behaves very similarly to MPL-S® for secretion of all the cytokines measured throughout the entire dose range. RC529 does show a much lower magnitude in the dose range effect with just small amounts of IFN-gamma and IL-12 p40 starting to show at the 100 ng/ml dose. However, at the 250 and 500 ng/ml doses RC529 induces an IL-2 level comparable to RC527 and MPL-S inductions.

Figure 10 was generated to compare the dose range effects of each of the lipid A mimetics as a percentage of the MPL-S® response. This allows a more direct comparison between the different lipid A mimetics’ effects relative to MPL-S® since the magnitude of cytokines elaborated was not consistent between experiments. For all the cytokines it would appear that RC511, RC527 and RC544 performed as well or better than MPL-S® as the dosage increased. It should be noted, however, that at the lowest dose tested of 10 ng/ml for RC511 and RC527 much higher levels of IFN-gamma and IL-12 p40 and to a lesser extent IL-2 are seen over MPL-S®. Yet as the dose increased to 50 ng/ml MPL-S® produced levels of IFN-gamma and IL-2 become comparable to RC511 and RC527. For IL-12 p40 secretion, however, not until almost the highest level tested of 500 ng/ml does MPL-S® perform as well as them. RC529, in contrast, appears to always induce lower amounts of than any of the other lipid A mimetics including MPL-S®. Although at the highest dose tested of 500 ng/ml it appears that RC511 approaches the MPL-S®
effects for IL-2 secretion levels it just reaches approximately 50% that of MPL-S® for IFN-gamma and IL-12 p40.
Figure 10. Relative dose range response from respective lipid A mimetics to that of the MPL-S® (@10, 50, 100, 250 & 500 ng/ml, left to right) caused secretion of IL-2, IFN-gamma and IL-12 p40 in the DO11.0 functional assay.
Discussion

For proper development of the DO11.10 \textit{in-vitro} functional assay it was considered important to create, as best as possible, optimal growth conditions for producing \textit{immature} bone marrow derived, murine DCs since it is the immature DC that is best at taking up antigen for eventual processing and presentation. As described in the Materials and Methods section, TGF\(\beta_1\) was added with GM-CSF for the 10 day DC culture. It has been shown that \textit{in-vitro} generation of DCs derived from CD34+ cord blood progenitors is promoted because TGF\(\beta_1\) protects the progenitor cells from apoptosis (63). An early protective effect (at 72 hours of culture) has been seen with TGF\(\beta_1\) as the proportion of apoptotic cells is reduced by more than 60\% in its presence (63). By day 7 of culture this correlates with an outgrowth of higher numbers and proportions of CD1a+ DC and reduced Fas/APO-1 expression on TGF\(\beta_1\) cultured cells (63). Thus, it was found that utilizing the 10 day tissue culture technique of Lutz et al. (46), in combination with adding a relatively low dose of TGF\(\beta_1\) to the culture containing GM-CSF, yielded high numbers of viable, nonadherent, \textit{immature} DCs from just a few mice.

In designing these series of experiments great lengths were also taken to utilize a source and grade of ovalbumin (see Materials and Methods) that had the lowest possible contaminating endotoxin levels so that the effects of the lipid A mimetics would be more truly measured and with the greatest sensitivity. Initial dose range studies (data not shown) indicated that as the concentration of ovalbumin for pulsing the immature DC was increased (eg. to 25 and 50 \text{ug/ml}) so did the background levels of IL-2 and IFN-gamma secretion stimulated by the antigen only, ovalbumin-pulsed DC controls in the DO11.10 functional assay. The 10\text{ug/ml} of ovalbumin used for pulsing in the data presented here yields very low baseline secretions of IL-2 and IFN-gamma for the ovalbumin antigen-only pulsed controls (see Figure 6). One interpretation is that it result in the combined effect of the lower potential endotoxin level contaminations in the amount of ovalbumin used for pulsing and the lower antigen amount actually used. As the concentration of ovalbumin is increased for pulsing the DC so are, in an antigen dose dependent manner, increasingly higher levels of IL-2 and IFN-gamma being secreted from the antigen-stimulated T cells. In fact, at a 50\text{ug/ml} ovalbumin dose the levels of IL-2 and IFN-gamma begin to approach the levels obtained when the same antigen-pulsed DCs are also subsequently treated with a maturation agent such as the lipid A compounds. Thus, the use of the maturation agent with the appropriate dose of antigen, i.e. not too high a level helps
provide two things for this in-vitro system. First, it provides a greater sensitivity for measuring the effects on DC surface phenotype and functional secretion of stimulated naïve T cells. And second, it allows for the dose range comparison of the effects of multiple lipid A compounds against the minimal effects caused by potentially, inextricable LPS contamination of the ovalbumin antigen.

In the DC and T cell syngeneic functional assay, CD3+ T cells were used which include both CD4+ and CD8+ T cells from the spleens of homozygous KJ1+, DO11.10 mice. In the DO11.10 mouse the vast majority of KJ1+ cells are CD4+ with 90% of these having the naïve phenotype (CD45RB<sup>high</sup>, L-selectin<sup>high</sup>) (64). They are considered naïve since they have never seen the ovalbumin antigen despite the fact that the vast majority has multiple copies of the T cell receptor for the processed, APC presented 323-339 peptide. A small population of CD4<sup>+</sup>, KJ1-26+ cells exist in the periphery but do not respond to ovalbumin (in-vivo) because they lack the CD4 co-receptor (suggesting that the CD8+ population also does not respond to ovalbumin processed or not). Thus, the predominant source of IL-2 and IFN-gamma in the DO11.10 functional assay is the CD4+ subset from the CD3+ selected population. In addition, it is the CD4+ T cell helper population, via the production of IL-2 and IFN-gamma, that can stimulate and sustain the proliferation of antigen specific cytotoxic T cells.

The ability of the ovalbumin specific CD4+ T cells to produce IL-2 and IFN-gamma can be attributed to the maturation of antigen-pulsed, murine bone marrow derived DC (bmdDC) by lipid A mimetics. The maturation of the DC after antigen loading may permit more potential for sustained co-stimulatory interaction between the DC as an APC and the naïve T cells. The use of maturation agents such as the lipid A mimetics also allows for the potential of more processed antigen to be presented on the surface of murine bmdDC. Class II, I-A<sup>d</sup>, molecules are constitutively expressed on murine bmdDCs as well as stored in secretory vesicles in preparation for their expression on the DC surface. Yet without the pro-inflammatory milieu provided by the lipid A, the antigen presenting capabilities of the DC are much less profound. Likewise, although ovalbumin may be processed and presented through I-A<sup>d</sup> to a significant

1 Previous work has suggested that the duration of antigenic stimulation determines the fate of both naïve and effector T cells (65). Naïve T cells may require 20 hours of sustained signaling whereas effector T cells may require less than one hour to become committed but die if the stimulation is too prolonged. Naïve and effector T cells show caparable capacity to engage TCRs but differ in their capacity to respond to different types of APCs. Both types of T cells, however, show similar dose response curves when antigen is presented by the DC.
degree, without the concomitant, significant upregulation of the co-stimulatory molecules CD80 and CD86 and CD40, the secretion of IL-2 and IFN-gamma by the naïve T cells can be still quite subdued if the DCs are not matured to increase the amounts of these important co-stimulatory molecules.

For example, the mean channel fluorescence, a measure of cell surface density, increased for both CD80 (>=4-fold) and class II, I-A^d (>2-fold) when the DCs were treated with all the lipids tested, except RC529 which produced less than 2-fold increases for each, compared to controls (i.e. no maturation and no antigen pulsed). Similarly, the cell surface densities of CD86 and CD40 are increased greater than 30% over the control when using the lipid A mimetics. An important exception, however, is that RC529 which induced very little increased cell surface expression of CD86 compared to no treatment controls and less than two thirds the expression of CD40 compared to all the other lipid A compounds. Thus, MHC class II molecule I-A^d, CD80, CD86 and CD40 are all expressed on the DC surface in much greater densities upon treatment with all the lipid A mimetics, except RC529, at their respective doses.

Similarly, in the DO11.10 functional assay the ovalbumin-only pulsed DC controls only yield hundreds of picograms per milliliter of IL-2 and IFN-gamma. However, upon lipid A treatment of antigen pulsed, bmd DCs there is a profound increase in these T cell produced cytokines with an increase in IFN-gamma secretion being even more profound than IL-2. LPS R595 at 10 ng/ml, MPL-S® and all the lipid A mimetics (except RC529) at the 100 ng/ml dose produced similar results above the control group. For IL-2 this difference can be described as a several hundred percent increase over the antigen pulsed control group, whereas for IFN-gamma the increase was several thousand percent.

LPS has also been shown to induce IL-12 production by macrophages and dendritic cells, which play key roles in the early development of TH1 responses (23,67). The results show (Table 4) that antigen-pulsed bmdDCs do produce, albeit variable amounts, of bioactive IL-12 p70 upon interaction with naïve DO11.10 T cells. However, the representative experimental data shown for IL-12 p70 levels (Table 4) yield values that may be lower and of a narrower range than could have been achieved if measured at much earlier stages of the DO11.10 T cell effector assay. The DCs used in this model were pulsed with ovalbumin with no washing out of that antigen and then matured overnight with the different maturation agents before washing out as much as possible the antigen and the respective maturation agent. For the sake of conveniently measuring multiple cytokines without jeopardizing the DC and T cell interaction a
very small aliquot was taken at 24 hours and again finally at 48 hours. Decreased production of IL-12 is an intrinsic property of human monocyte-derived DCs subsequent to maturing with IL-1 beta and TNF-alpha (68). LPS and the lipid A mimetic compounds have been shown to induce TNF-alpha and IL-1 beta by DC (data not shown) within the same timeframe in which the DCs were matured. These early secreted cytokines could have contributed to DCs’ decreased production of IL-12 p70 at the 48 hour time point in the DO 11.10 functional assay. Moreover, in the DCs’ interaction with CD4+ T helper cells, it has been found that the highest amounts of secreted IL-12 p70 are found within two to four hours (>100 pg/ml) subsequent to initiating maturation of DCs whereas it was significantly diminished at 12 and 48 hours (<10 pg/ml) (68). The experimental results reported herein for secreted IL-12 p70 were from 48 hour supernatants- perhaps too late for measuring peak secretion levels. Nonetheless, stimulation with LPS R595 at 10ng/ml in-vitro resulted in similar or greater amounts of production of IL-12 p70 (as well as IFN-gamma and IL-12 p40) compared to ten-fold higher doses of all the lipid A compounds. Moreover, the amounts of secreted IL-12p70 varied between experiments (data not shown) but, generally speaking, the lipid A mimetics (with the exception of RC529) and even MPL-S® in some experiments caused appreciable amounts of IL-12 p70 to be secreted in the DO11.10 functional assay.

It has been demonstrated that LPS and IFN-gamma act synergistically in the induction of IL-12 and that IFN-gamma has an additive effect with LPS on IL-12 p40 induction in human monocytes (69,70). MPL-S® has been shown to have a synergistic effect with IFN-gamma as evidenced by a 2 and 3-fold increase in IL-12 p40 and IL-12 p70 in-vitro productions, respectively, compared to treatment of naïve murine spleen cells with MPL-S® alone (71). In this study, however, the lipid A compounds may have been removed from the potential milieu of the DC: T cell interaction when the DCs were washed after antigen pulsing and lipid A treatments before being put in the presence of the naïve T cells in the DO11.10 functional assay, thus, diminishing potential synergy with any newly secreted IFN-gamma. Additionally, IL-12 p40 is constitutively expressed by DCs and has been found to be released by direct stimulation of the murine bmdDC by lipid A compounds (unpublished data).

In summary, there are basically three advantages that the maturation induced by lipid A compounds have upon the dendritic cell, the only professional antigen presenting cell capable of educating naïve T cells. One, classical co-stimulatory molecules on the cell surface of DC such as CD80 and CD86,
CD40 receptor and the MHC class II cell surface molecule I-A<sup>d</sup> found on the dendritic cell are all upregulated by maturation of the DC. As the flow cytometry data shows the percentage and densities of all of these important DC surface markers, that directly interact with T cells, are significantly increased by using lipid A compounds as a maturation agent for the antigen-pulsed, immature DC. Two, this directly coincides with and supports the observed in-vitro secreted cytokine data also presented in this thesis. The synthetic lipid A mimetics chosen for this study, in addition to LPS and its attenuated natural derivative MPL-S®, potentiate the functional development of antigen loaded DCs as shown by their enhanced ability to stimulate naïve, antigen-specific T cells to secrete IL-2 and IFN-gamma in the DO11.10 model.

Third, there are different potencies of the lipid A mimetics, as expressed as a percentage of the MPL-S® response. When RC511 and RC527 are used to mature antigen-pulsed DCs they induce the secretion of relatively larger magnitudes of IL-2, IFN-gamma and IL-12 p40 especially at the lower doses (10ng/ml) of the dose range compared to MPL-S® and the other lipid A mimetics (Figure 10). Whereas, at the highest doses tested (50 to 500 ng/ml) the DCs maybe not be additionally enhanced as antigen-presenting cells since all of the lipid A mimetics tested appear to approach that of MPL-S®. In fact, for RC511 at the 500 ng/ml level the IFN-gamma secretion is about half that of MPL-S® suggesting that the DCs maybe refractory at the highest concentrations tested. This suggests that for RC511 (especially for IFN-gamma) and RC527 (Figures 8 and 9), at least, lower dose range studies are needed to test the potency and efficacy of this promising lipid A mimetic family of compounds. One exception, however, is RC529 which clearly does not induce high cytokine levels compared to all the other compounds except, perhaps, at the 250 and 500 ng/ml level for IL-2 secretion.

Finally, these data were produced from bone marrow derived DCs in the context of an antigen-specific model. It is not practical, however, to use human DCs derived from bone marrow and the decision of which cancer specific antigens to use is difficult. Nonetheless, the innate abundance of DC monocyte precursors found in peripheral blood, (barring any prior exposure to corticosteroids which have been found to negatively impact the maturation and function of DCs (72)) may help provide the real possibility of ex-vivo immunotherapy in a multiple vaccine regimen. As another modality towards the successful management of cancer through immunotherapy, autologous DCs can be pulsed with promising tumor specific antigen(s) candidates and then matured, for example, using clinically-tested and well-tolerated
MPL-S®, before reinfusion back into the patient perhaps affording clinically favorable, therapeutic CD4+ T helper cell and effector cytotoxic T cell functions.
REFERENCES


