May 1998

Tnf(α)-dependent and Tnf(α)-independent Activation of Macrophage Effector Function

Annette R. Clemons-miller

East Tennessee State University

Follow this and additional works at: http://dc.etsu.edu/etd
Part of the Immunology and Infectious Disease Commons

Recommended Citation

This Dissertation - Open Access is brought to you for free and open access by Digital Commons @ East Tennessee State University. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of Digital Commons @ East Tennessee State University. For more information, please contact dcadmin@etsu.edu.
INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6” x 9” black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
TNFα-DEPENDENT AND TNFα-INDEPENDENT ACTIVATION OF MACROPHAGE EFFECTOR FUNCTION

A Dissertation
Presented to
the Faculty of the Department of Microbiology
James H. Quillen College of Medicine
East Tennessee State University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Biomedical Sciences

by
Annette R. Clemons-Miller
December 1998
APPROVAL

This is to certify that the Graduate Committee of

ANNETTE R. CLEMONS-MILLER

met on the

Eleventh day of November, 1998

The committee read and examined her dissertation, supervised her defense of it in an oral examination, and decided to recommend that her study be submitted to the Graduate Council, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.

Chair, Graduate Committee

Signed on behalf of the Graduate Council

Dean, School of Graduate Studies

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
ABSTRACT

TNFα-DEPENDENT AND TNFα-INDEPENDENT ACTIVATION OF MACROPHAGE EFFECTOR FUNCTION

by

Annette R. Clemons-Miller

Tumor necrosis factor α (TNFα) is a pleiotropic cytokine that is predominantly produced by activated macrophages. The effects of TNFα are as diverse as the cells with which it interacts, e.g., stimulating fibroblast growth, exerting cytotoxic/cytostatic activity against various human and murine cell lines, promoting inflammation through upregulation of endothelial adhesion molecules and IL-8 production. Yet TNFα is best known, and in fact was originally described, for its role in the bacterial-induced hemorrhagic necrosis of tumors and exacerbation of septic shock in which aberrant TNFα production leads to vascular collapse, cachexia, multiple organ failure, and ultimately death in as many as 100,000 people each year in the United States alone.

LPS, a component of the outer cell wall of gram-negative bacteria, is the principal inducer of macrophage TNFα production. TNFα production can be enhanced by IFNγ which also induces upregulation of TNFα receptors allowing for the establishment of a TNFα autocrine loop. It has been hypothesized that autocrine TNFα stimulation plays a critical role in the induction of macrophage effector function, e.g., nitric oxide production. This dissertation represents efforts to evaluate the respective roles of the TNFα receptors in the induction of macrophage effector function, in addition to examining the mechanism by which autocrine TNFα exerts its effects on macrophages.

Exploiting the species specificity of the murine TNFα receptor type 2 (TNF-R2), splenic macrophages were stimulated with human TNFα (which binds to TNF-R1 but not TNF-R2), in the presence of IFNγ. Human TNFα was effective in the induction of nitric oxide production, albeit at concentrations 12.5-fold greater than those required by murine TNFα to achieve the same result. Addition of anti-TNF-R1 completely inhibited the murine TNFα mediated induction of macrophage effector function. However, treatment with anti-TNF-R2 resulted in partial inhibition of macrophage activation. Taken together this data suggests that the primary TNFα mediated signals involved in macrophage activation are transduced through TNF-R1, although TNF-R2 appears to contribute to the intensity of the macrophage response.

To evaluate the role of autocrine TNFα signaling in the induction of macrophage effector function, immortalized macrophages from normal C57Bl/6J mice (B6/J2) and C57Bl/6J mice containing gene targeted disruptions of the TNF-R1 and TNF-R2 genes (TRN) were...
stimulated under CD14-dependent and CD14-independent conditions. Although the B6/J2 and TRN clones mounted similar NO responses to LPS in the presence of serum, the TRN macrophages generated a weak nitric oxide response as compared to B6/J2 when stimulated with LPS under serum-free conditions. The involvement of TNFα autocrine stimulation in the CD14-independent activation was corroborated by the ability of soluble TNF-R1 to inhibit the response of B6/J2 macrophages to LPS in serum-free medium. CD14-independent LPS stimulation of TRN and B6/J2 resulted in equivalent levels of IL-1β, TNFα, and iNOS gene expression, as determined by RT-PCR, and in release of equivalent amounts of biologically active TNFα. However, western blot analysis revealed that iNOS protein production by TRN was as much as 50% less than that produced by B6/J2. These results indicate that autocrine TNFα stimulation contributes to the signaling pathways initiated by ligation of CD14-independent LPS receptors and may be involved in iNOS post-transcriptional regulation.
ACKNOWLEDGMENTS

I would like to thank Dr. Robert Stout for taking me on as one of his graduate students. As I am sure he would tell you, I have probably not been one of his most productive students but I hope he has enjoyed working with me as much as I have enjoyed working with him. He's taught me not only the ethics and responsibilities of being a respected researcher but also how to function in the political realm of academia.

To the remaining members of my committee: Dr. Jill Suttles, Dr. Rob Schoborg, Dr. David Chi, and Dr. Krish thank you first and foremost for helping me graduate and get reacquainted with my husband!!! I also want to thank each of you for all the technical advice you've given me, I just wish the *!?@? T cells would have cooperated. In addition, I'd like to extend a special thanks to Jill for convincing me to come to ETSU, it was with out a doubt the best decision I've ever made.

I'd also like to acknowledge two people who were instrumental in my pursuit of this degree, Dr. Dru Henson and Dr. Richard Henson. First, a "yes, you told me so" to Dr. Richard Henson because he saw in me the potential to be more than I thought I could be. To "Momma" Henson thank you for introducing me to the field of Immunology and inspiring me to join this crazy pursuit of knowledge. My hope is that some day I can have your job!

To Debbie, John, and Lori my desk and chair are now up for grabs! I will miss all the late afternoon discussions of philosophical, political, and of course sports related issues (e.g. who are UT and Denver going to defeat this weekend!)

To the new kids in the Suttles lab: take good care of my cell lines. Clark keep up the good work, someday you'll be an excellent gel photographer. Denise I am glad to have had the last several months to get to know you, I only wish they would have moved the lab over here sooner. Bob you've been a great help to me over the past five years, especially in teaching me to be patient with T cell maintenance and instructing me in the finer art of FACS analysis....believe me, the HIM ARE CD40L NEGATIVE!

Finally, to my family a heartfelt thank you for all of your encouragement and support with what has amounted to a career as a professional student. After 22 years I am graduating for the LAST time! A special thanks goes to my wonderful in-laws for giving me a place to stay for the last five months, I'll miss you both but am anxious to rejoin Rob in Little Rock. As for Rob, I am finally able to do what you keep asking me to do every time we talk on the phone...... I AM GRADUATING!!!! Thank you so much for all your love and support especially during these last few months. Keep the light on, I'll be home soon!
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>APPROVAL</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>xi</td>
</tr>
</tbody>
</table>

## CHAPTER

1. INTRODUCTION ................................................................. 1
   - Macrophage Origin and Functions ........................................ 1
   - TNFα: The Molecule .......................................................... 6
   - TNFα: The Receptors ......................................................... 8
   - TNFα: The Autocrine Loop ................................................. 10

2. MATERIALS AND METHODS .................................................. 13
   - Reagents ........................................................................... 13
   - Endotoxin Screening ....................................................... 13
   - Media ................................................................................ 14
   - Antibodies ........................................................................ 14
   - Animals ............................................................................. 15
   - Cell Lines .......................................................................... 15
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage Cultures</td>
<td>15</td>
</tr>
<tr>
<td>Generation of TNFR-1/TNFR-2 -/- Macrophage Lines</td>
<td>16</td>
</tr>
<tr>
<td>TNFα Assay</td>
<td>17</td>
</tr>
<tr>
<td>Nitrite Assay</td>
<td>17</td>
</tr>
<tr>
<td>Oxidative Burst Assay</td>
<td>17</td>
</tr>
<tr>
<td>Cell-ELISA</td>
<td>18</td>
</tr>
<tr>
<td>FACS Analysis</td>
<td>19</td>
</tr>
<tr>
<td>RT-PCR Analysis</td>
<td>19</td>
</tr>
<tr>
<td>Western Blot Analysis</td>
<td>20</td>
</tr>
</tbody>
</table>

3. ROLE OF TNFα RECEPTORS IN MACROPHAGE ACTIVATION                      | 22   |
| Effect of Murine vs. Human TNFα in the Induction of Macrophage Nitric Oxide Production | 22   |
| Relative Expression of TNF-R1 and TNF-R2 on RAW 264.7 and C57BL/6J Macrophages | 24   |
| Antagonistic Effect of Anti-TNF-Receptor Antibodies on Macrophages     | 24   |
| Effect of IFN-γ and GM-CSF Pretreatment on Induction of Macrophage Effector Function | 29   |

4. LPS STIMULATION OF TNFα-RECEPTOR DEFICIENT MACROPHAGES              | 33   |
| Generation and Characterization of Wild-Type and TNF-Receptor Negative Macrophage Lines | 33   |
| Involvement of the TNFα Autocrine Loop in the Induction of Macrophage NO Production Under Serum-Free Conditions | 38   |
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>The Many Functions of Macrophages</td>
<td>2</td>
</tr>
<tr>
<td>2.</td>
<td>Cartoon Depiction of T Cell/Macrophage Contact Dependent Interactions</td>
<td>5</td>
</tr>
<tr>
<td>3.</td>
<td>Model for TNFα Binding to TNFα Receptors</td>
<td>9</td>
</tr>
<tr>
<td>4.</td>
<td>Species Specificity of the Murine TNF-Receptor 2</td>
<td>10</td>
</tr>
<tr>
<td>5.</td>
<td>Cartoon of the Identified TNF-Receptor Associated Signaling Molecules</td>
<td>11</td>
</tr>
<tr>
<td>6.</td>
<td>Effect of Murine TNFα vs. Human TNFα on the Induction of Macrophage Effector Function</td>
<td>23</td>
</tr>
<tr>
<td>7.</td>
<td>Relative Expression of TNF-R1 and TNF-R2 on RAW 264.7 and C57Bl/6J Macrophages</td>
<td>25</td>
</tr>
<tr>
<td>8.</td>
<td>Titration of Anti-TNF-Receptor Antibodies on Macrophages</td>
<td>26</td>
</tr>
<tr>
<td>9.</td>
<td>Antagonistic Effect of Anti-TNF-Receptor Antibodies on Macrophages</td>
<td>28</td>
</tr>
<tr>
<td>10.</td>
<td>Effect of Cytokine Priming on the TNFα-Mediated Induction of Macrophage Effector Function</td>
<td>30</td>
</tr>
<tr>
<td>11.</td>
<td>Effect of Cytokine Priming on the TNFα-Mediated Induction of Effector Function in RAW 264.7</td>
<td>32</td>
</tr>
<tr>
<td>12.</td>
<td>Surface Phenotyping of Immortalized Macrophage Lines</td>
<td>34</td>
</tr>
<tr>
<td>14.</td>
<td>Assessment of Functional Characteristics via Induction of Nitric Oxide Production</td>
<td>37</td>
</tr>
<tr>
<td>15.</td>
<td>Involvement of TNFα in the Induction of Nitric Oxide Production Under Serum-Free Conditions</td>
<td>39</td>
</tr>
</tbody>
</table>
Figure Page

16. LPS Induced Gene Expression in Serum-Free Media I .............. 40
17. LPS Induced Gene Expression in Serum-Free Media II .............. 41
18. Induction of TNFα Production Under Serum-Free Conditions ........ 42
19. LPS Induced iNOS Protein Expression Under Serum-Free Conditions ................................................................. 44
20. Soluble Murine TNF-R1 Inhibition of B6/J2 Nitric Oxide Production ................................................................. 45

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>hTNFα</td>
<td>Human TNFα</td>
</tr>
<tr>
<td>mTNFα</td>
<td>Murine TNFα</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>TNF-R1</td>
<td>TNFα receptor type 1</td>
</tr>
<tr>
<td>TNF-R2</td>
<td>TNFα receptor type 2</td>
</tr>
<tr>
<td>B6/J2</td>
<td>Wild-type immortalized macrophage line</td>
</tr>
<tr>
<td>TRN</td>
<td>TNF receptor deficient immortalized macrophage line</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO₂</td>
<td>Nitrite</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum-free media</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-monocyte colony stimulating factor</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>PEC</td>
<td>Peritoneal elicited cells</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

Macrophage Origin and Functions

Macrophages are derived from myelocytic precursors in the bone marrows that initially differentiate into circulating monocytes that eventually migrate into the extravascular tissues and terminally differentiate into mature macrophages. Monocyte migration into the tissues appears to be a random event with macrophages found throughout the body including the liver (Kupffer cells), lung (alveolar macrophages), connective tissue (histiocytes), spleen, bone (osteoclasts), central nervous system (microglia), skin (Langerhans' cells), peritoneal and pleural cavities. Interestingly, macrophages appear to be a product of their environment, that is to say that the functional, morphological, and phenotypic heterogeneity of a certain macrophage population reflects the local conditions to which they are subjected during their maturation.

Macrophages were first characterized by Elie Metchnikoff in the late 1800s as mediators of harmonization. The basic tenet of his theory still holds true in that many of the roles of macrophages revolve around maintaining “harmony” within the immune system. These roles can be divided into five generic functional activities encompassing both innate and acquired immunity: phagocytosis, effector function, inflammatory function, anti-inflammatory function, and accessory function (Figure 1).
One of the major effector functions displayed by macrophages is the production of reactive nitrogen intermediates. The generation of reactive nitrogen intermediates, specifically production of nitric oxide, involves the induction of a calcium-independent macrophage isoform of nitric oxide synthase, inducible nitric oxide synthase (iNOS)\textsuperscript{5,6}. iNOS is a NADPH-dependent, cytosolic enzyme that catalyzes the oxidation of the terminal guanido-nitrogen of L-arginine resulting in the formation of stoichiometric amounts of L-citrulline and nitric oxide (NO)\textsuperscript{7,8}. Stimulation of macrophages with either LPS or TNFα in the presence of IFNγ results in the tightly regulated transcription of iNOS synthesis\textsuperscript{9,10}. Transcription of iNOS is controlled through binding of specific DNA binding proteins to two regions (region I and II) in the promoter/enhancer elements of the iNOS gene. Region I and II have been shown to be necessary for the LPS-activated expression iNOS, whereas region II alone was found to mediate IFNγ regulation and acted primarily as an enhancer of iNOS induction\textsuperscript{11}. Both regions were found to contain
binding sites for numerous DNA binding proteins. Specifically, consensus binding
sequences for NF-IL6, NF-κB, and TNF-REs were found within region I, while IFN-
responsive elements (e.g., IRF-1) were concentrated in region II 11.

Once induced, iNOS is capable of producing locally high levels of NO, far greater
than those produced by other nitric oxide synthase isoforms, making it a major effector
molecule in the defense against certain tumor cells, parasitic fungi, protozoa, helminths,
and mycobacteria 12. However, the toxic levels of NO are not only effective against
microbicidal invaders but are also capable of damaging normal host tissues, potentially
contributing to autoimmune tissue destruction 13. NO appears to exert its effects through
DNA damage or the inactivation of cellular metabolic pathways. Specifically, NO can
react with the iron-sulfur-containing enzymes, such as aconitase and complexes I and II of
the mitochondrial electron transport chain, resulting in the formation of nitrosyl iron
complexes, rendering the enzyme inactive 12,13. Thus, the production of NO by activated
macrophages is a major effector molecule employed in the nonspecific aspect of the
immune response.

The inflammatory and anti-inflammatory functions of macrophages primarily
involve the production of specific immunomodulatory cytokines. The major inflammatory
cytokines produced by macrophages include TNFα, IL-1, and IL-6 14. These three
cytokines constitute the basis of the proinflammatory cascade in which IL-1 and TNFα can
not only enhance the production of themselves and each other, but both can also enhance
the production of IL-6 4. The effects of IL-1 are seemingly limitless: it is chemotactic,
pyrogenic, induces the expression of selectins and intercellular adhesion molecules
(ICAMs) on vascular endothelium, induces the production of collagenase, metalloproteinases, and PGE₂, and stimulates bone and cartilage resorption. TNFα, which will be discussed in detail later, is as intimately involved in the inflammatory response as IL-1, often with their effects overlapping each other. The anti-inflammatory aspect of macrophage function involves another set of immunomodulatory cytokines, which include TGF-β, IL-10, and the IL-1 receptor antagonist (IL-1Ra).

TGF-β can exert both stimulatory and inhibitory effects. The stimulatory effects include the enhancement of matrix protein synthesis by fibroblasts, osteoblasts, and endothelial cells, induction of osteoblast proliferation, fibroblast chemotaxis, and the production of collagenase, all of which play a role in fibrosis and the promotion of wound healing. TGF-β has also been shown to inhibit not only all known IL-2 effects, but also inhibits the actions of several other cytokines including the activation of macrophages by IFNγ. IL-10, which is produced not only by macrophages but also by mast cells, T cells, B cells, and keratinocytes, is an immunosuppressive agent that inhibits proinflammatory cytokine production (e.g., IL-1, TNFα, IL-6), reduces macrophage expression of class II MHC molecules, and reduces nitric oxide production. The IL-1Ra, which can be induced by either IL-1 or TNFα, appears to function primarily as a feedback inhibitor in the production of IL-1 and TNFα.

Thus far, the macrophage functions discussed revolve around the macrophage's role in nonspecific (innate) immunity. However, accessory function, the fifth and final macrophage function to be discussed, describes the macrophage's role in acquired immunity.
As accessory cells, macrophages perform not only their classical function of antigen presentation but also assist in T cell activation through contact-dependent and contact-independent mechanisms. The contact-independent mechanisms involve the production of costimulatory cytokines such as IL-1, TNFα, and IL-6. The contact-dependent mechanism, as the name implies, involves surface to surface interaction between the T cell and macrophage. To enable this conjugation the macrophage expresses ligands (e.g., CD80, CD86) and receptors (e.g., CD40, LFA-1) that are specific for receptors (e.g., CD28, CD152) and ligands (e.g., CD154, I-CAM) on the T cell (Figure 2).

**Figure 2.** Cartoon Depiction of T Cell/Macrophage Contact Dependent Interactions.

Recent studies have suggested that the contact-dependent signaling between T cells and macrophages is a reciprocal event— that is, not only does the macrophage provide cognate co-stimulation for the T cell, but it is also the recipient of cognate co-stimulation from the T cell. The contact-dependent, reciprocal activation of macrophages by T cells was best illustrated by studies in which either paraformaldehyde-fixed or
purified membrane preparations from activated T cells were capable of inducing macrophages/monocytes to produce either NO or IL-1 and TNFα. The T cells used in the studies above were activated to induce the expression of CD154 (CD40L) prior to addition to the macrophage cultures. CD154, a transiently expressed protein that is maximally expressed following 5-10 hrs of activation, binds to CD40 which is functionally expressed on B cells, vascular endothelial cells, epidermal dendritic cells, fibroblasts, and monocytes/macrophages. The involvement of CD40:CD154 interactions in humoral immune responses (e.g., B cell activation and differentiation, immunoglobulin class switching) has been well established. While the involvement of CD40:CD154 in cell-mediated inflammatory responses such as multiple sclerosis and experimental allergic encephalitis has only recently begun to be investigated, the possibility of CD40:CD154 induced TNFα participating in the autocrine activation of macrophages/monocytes has yet to be addressed.

**TNFα: The Molecule**

TNFα was originally described by its ability to cause hemorrhagic necrosis and was found to be identical to the mononuclear cell-derived factor “cachectin”, which had been shown to mediate shock in gram-negative sepsis. In addition to septic shock, aberrant TNFα production has been shown to be involved in the exacerbation of cerebral malaria, bacterial meningitis, graft-versus-host disease, and a number of autoimmune diseases. Under “normal” circumstances, TNFα plays an important role as an immunostimulator and mediator of host resistance to many infectious agents.
TNFα is produced predominantly by activated macrophages but can also be produced by a number of other cell types including endothelial cells, smooth muscle cells, astrocytes, CD4+ and CD8+ T cells, keratinocytes, mast cells, and dendritic cells. The effects of TNFα are as diverse as the cells with which it interacts—e.g. stimulating fibroblast growth by enhancing the expression of c-myc and c-fos, exerting cytotoxic/cytostatic activity against various human and murine cell lines, and promoting inflammation by inducing endothelial cells to secrete IL-8 and to express adhesion molecules such as ELAM-1 and ICAM-1. To date no specific TNFα-induced biologic activity has been defined that is common to all TNFα-responsive cells.

TNFα, the patriarchal member of the TNFα superfamily, can interact with its target cells either as a transmembrane or soluble protein. TNFα, along with other members of the TNFα superfamily, is synthesized as a type II transmembrane protein characterized by the extracellular C-terminus. The secreted form is generated by proteolytic cleavage of the 26 kDa membrane bound protein by the 85 kDa TNFα converting enzyme (TACE). The cleavage event results in the release of a 17 kDa monomer that normally circulates as a noncovalently bound homotrimer.

The three-dimensional shape of the TNFα homotrimer has been described as a triangular cone. Each of the three subunits has a typical jelly roll-β structure characterized by the two β-pleated sheets and five anti-parallel β-strands. While the outside β-sheet is rich in hydrophilic residues, the inner sheet is hydrophobic and contains the C-terminal segment located close to the central axis of the trimer. Mutational analyses have mapped the active region of the TNFα molecule to the lower half of the triangular pyramid.
in the groove created between each of the subunits which corresponds to the receptor binding domain\textsuperscript{54,55}.

\textbf{TNFα: The Receptors}

Two receptors have been characterized that show high binding affinity for TNFα, TNFα-receptor type 1 (TNF-R1) and TNFα-receptor type 2 (TNF-R2), which bind TNFα with $K_d$ values of 0.5 nM and 0.1 nM, respectively\textsuperscript{56}. TNF-R1 and TNF-R2 belong to a family of receptors referred to as the TNFα receptor superfamily which consists not only of the TNFα receptors but also LT-β receptor, Fas (CD95/Apo1), CD40, CD30, CD27, p75 NGF receptor, OX-40, 4-1BB, mediator of herpes simplex virus entry, and death domain-containing receptor 3 (DR3)\textsuperscript{57}. This superfamily is characterized by an extracellular domain that can be divided into four roughly repeating cysteine-rich domains which share significant intersubunit sequence homology\textsuperscript{38,48,58}. Noting the trimeric structure of the members of the TNFα superfamily, it has been suggested that the receptors in the TNFα receptor superfamily undergo receptor clustering during signal transduction\textsuperscript{59,60} (Figure 3).

Homology between the two TNFα receptors, 20% amino acid sequence identity within the extracellular domain and 5% within the cytoplasmic domain\textsuperscript{61}, is not any greater than between other members of this family\textsuperscript{48}. The lack of homology within the intracellular domains of TNF-R1 and TNF-R2 suggests that each receptor uses a different signaling pathway\textsuperscript{62}. Stimulation through TNF-R1 has been linked to the activation of
NF-κB, accumulation of mRNA for c-fos and IL-6, surface expression of IL-2R and HLA Class I and Class II, inhibition of cell growth, and cytotoxicity. In contrast, TNF-R2 has been shown to be involved in the TNFα-dependent proliferative responses of peripheral blood lymphocytes and thymocytes, induction of GM-CSF secretion, inhibition of early hematopoiesis, and the upregulation of costimulatory molecules.

The cloning of the two murine TNFα receptors demonstrated that although TNF-R1 had a similar affinity for both recombinant mTNFα and hTNFα, TNF-R2 showed affinity for only mTNFα, thus defining TNF-R2 as a species-specific receptor (Figure 4). The ability of hTNFα to bind to TNF-R1 but not to TNF-R2 provides a model allowing separation of TNF-R1 mediated effects from TNF-R2 effects. Using this model, TNF-R1 was found to mediate most of the biological effects of TNFα including initiation.
of cellular cytotoxicity\textsuperscript{62,68,69} and apoptosis\textsuperscript{70,71}, enhancement of oxidative burst\textsuperscript{59,72}, as well as mediation of systemic shock during sepsis\textsuperscript{67,73,74}.

Like many other cytokine receptors, TNF-R1 and TNF-R2 do not contain tyrosine kinase domains within their intracellular domains\textsuperscript{75}, thus the mechanism(s) by which the two receptors initiate their respective signal cascades has remained cryptic. The lack of intrinsic tyrosine kinase activity generally suggests that ligand-receptor interactions result in receptor clustering leading to association of active signaling molecules with the receptor\textsuperscript{76}. Through the implementation of the yeast-two hybrid screening method\textsuperscript{77}, several such TNF receptor-associated molecules have been identified and are depicted in Figure 5. Of the TNF receptor associated molecules that have been studied, most appear to be involved in the activation of \textit{NF-κB} and/or the induction of apoptosis\textsuperscript{57}.

\begin{figure}
\centering
\includegraphics[width=0.8\linewidth]{figure4.jpg}
\caption{Species Specificity of the Murine TNF-Receptor 2.}
\end{figure}

\textbf{TNFα Autocrine Loop}

The production of TNFα by macrophages and their concomitant expression of TNFα receptors implies that TNFα autocrine stimulation could play a critical role in the
induction of macrophage effector function, such as nitric oxide production\textsuperscript{78-81}. This role has been supported by studies in which anti-TNFα neutralizing antibodies inhibited the ability of LPS and IFNγ to induce macrophage effector functions\textsuperscript{82-85}, as well as the observation that recombinant TNFα, in the presence of IFNγ was sufficient for the induction of macrophage effector function\textsuperscript{84}. Noting the critical role that autocrine TNFα plays in the LPS-mediated induction of macrophage effector function, it is conceivable that TNFα activates the signaling pathway(s) that are crucial for the synergistic response to LPS and IFNγ.

\textbf{Figure 5.} Cartoon of the Identified TNF-Receptor Associated Signaling Molecules.
This dissertation was designed not only to evaluate the respective roles of the two TNFα receptors in the induction of macrophage effector function, but also to examine the mechanism by which TNFα exerts its effects on macrophages. Specifically, the question of the involvement of a TNFα autocrine loop in the induction of macrophage activation has been addressed utilizing macrophage lines with targeted disruptions of the TNFα receptors.

Gaining an understanding of the means by which TNFα activates macrophage effector function, and through which TNFα receptor those effects are mediated will allow for potential targets for intervention and regulation of aberrant TNFα production.
CHAPTER 2
MATERIALS AND METHODS

Reagents

Recombinant murine TNFα, human TNFα, and murine GM-CSF were obtained from Genzyme Corp. (Cambridge, MA). The specific activity reported by Genzyme was \(6.67 \times 10^3\) U/ng for murine TNFα, \(5.56 \times 10^2\) U/ng for human TNFα, and \(7.8 \times 10^4\) U/ng for the GM-CSF. Recombinant murine IFNγ was acquired from Amgen Biologicals (Thousand Oaks, CA) and R&D Systems (Minneapolis, MN). Recombinant soluble murine TNFα-receptor type I was obtained from R&D Systems (Minneapolis, MN). Bacterial lipopolysaccharide (LPS; from \(E. coli\) O111:B4-W) and gentamicin were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS; prescreened for endotoxin levels of \(<0.05\ ng/ml\) was purchased from HyClone Laboratories, Inc. (Logan, UT) and Atlanta Biologicals (Norcross, GA).

Endotoxin Screening

All tissue culture reagents used were either certified as containing no, or low, endotoxin when purchased, or were assayed for endotoxin concentration by chromogenic limulus assay (Sigma). Stock solutions containing \(>1\ ng/ml\) (10 EU/ml) were considered unsuitable for use. Stock solutions were diluted in assays such that endotoxin levels did not exceed \(1\ pg/ml\).
Media

Complete culture media for macrophage activation (R5 + 2-ME) contained RPMI 1640 with L-glutamine (HyClone Laboratories), supplemented with 5% heat-inactivated FBS (HI-FBS), 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Sigma Chemical Co.), and 50 μM 2-mercaptoethanol (Sigma Chemical Co.). The culture medium for generation of macrophages from murine spleen cells (R5) was the same as the macrophage activation media described above except 2-mercaptoethanol was not included. Serum-free media was obtained from Gibco-BRL (Grand Island, NY).

Antibodies

Purified polyclonal antisera to murine TNF-R1 and TNF-R2 were generously provided by Genentech, Inc. (San Francisco, CA). Phycoerythrin-labeled monoclonal rat anti-mouse TNF-R1 and TNF-R2 were obtained from Caltag Laboratories (Burlingame, CA). Fluorescein conjugated NeutraLite avidin was obtained from Molecular Probes (Eugene, OR).

The following monoclonal antibodies (mAbs) were collected from culture supernatants from the appropriate hybridomas: rat anti-mouse Mac-1 α6, anti I-A b,d (28-16-8S) β7, rat anti-mouse CD4 (GK-1.5) γ8, rat anti-mouse CD8 (53-6.72) δ9, rat anti-mouse ThB (49h4) 90, and the rat anti-mouse macrophage (F4/80) 91 (American Type Culture Collection, Rockville, MD). The anti-MAC-1 was purified over a protein G-sepharose 4B affinity column (Pharmacia LKB Biotech, Inc., Piscataway, NJ) followed by dialysis and sterile filtration prior to use.
**Animals**

Inbred female C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME), while breeding pairs of the TNF-R1/TNF-R2 homozygous double knockout mice were provided by Genentech, Inc. (San Francisco, CA). Only mice between the ages of 6 and 14 weeks were used for experimentation.

**Cell Lines**

The macrophage line RAW 264.7 and L929 fibroblasts were obtained from American Tissue Type Culture Collection (Rockville, MD). iCRE/J2 was generously donated by Dr. George Cox (NIH, Bethesda, MD).

**Macrophage Cultures**

Splenic macrophages were generated as previously described in detail. Briefly, spleen cells resuspended to 4-4.5 x 10^6/ml in R5 were added to a 96-well microtiter culture plate in 0.2 ml aliquots. Following a culture period of 5 days, nonadherent cells were removed by washing with Dulbecco's phosphate-buffered saline (DPBS) supplemented with 2% HI-FBS. Peritoneal cells were obtained following peritoneal lavage of the mice with a 4:1 PBS/Citrate (3.4%) buffer. The peritoneal cells were then resuspended to 1 x 10^6/ml in R5, added to a microtiter plate in 0.2 ml aliquots, and allowed to adhere overnight. RAW 264.7 maintained in R5 were harvested and resuspended to 1 x 10^6/ml and added to a microtiter plate in 0.2 ml aliquots,
and allowed to adhere overnight. The virally transformed macrophage lines and/or clones were maintained in R5.

**Generation of TNFR-1/TNFR-2 -/- Macrophage Line**

J2-immortalized macrophage lines were generated from both C57Bl/6J (B6/J2) and TNFR-1/TNFR-2 -/- (TRN) mice as previously described. Briefly, density gradient purified bone marrow (Lympholyte-M, Atlanta Biologicals) from both hind limbs was resuspended in 5 ml of CRE/J2 supernatant supplemented with 5% HIFBS, which had been filtered through a 0.45 μm filter, and placed into a Nunc™ T-25 vented cap flask (Fisher Scientific). Polybrene (Sigma Chemical Co.) and murine GM-CSF were added to the culture at final concentrations of 5 μg/ml and 10 ng/ml, respectively. Following an overnight incubation, the media was removed and replaced with fresh R5 supplemented with an additional 5% HIFBS and 10 ng/ml GM-CSF. After 7 days of culture the supernatant was discarded and the cells were rinsed gently with R5 and placed back in culture in R5 with GM-CSF. Macrophage clones were then obtained by limiting dilution. Once well established, the clones were maintained in R5 without GM-CSF. The resulting cells were then assayed for macrophage distinctive surface phenotype (MAC-1+, F4/80+) and for macrophage function.

**TNFα Assay**

Bioactivity of TNFα in the culture supernatants was determined by the L929 bioassay as previously described. L929 fibroblasts treated with 4 μg/ml actinomycin D
were plated at $5 \times 10^4$ cells/well with or without culture supernatants and incubated at 37°C overnight. The supernatant was then removed and replaced with 100µl 0.2% crystal violet. After 5 min, the plate was rinsed with tap water 6-7 times and blotted dry. To each of the wells, 100µl 1% SDS (Fisher Scientific) was then added. The plate was shaken and the OD₅₅₀ read using an automated plate reader. TNFα concentration was calculated from a standard curve.

**Nitrite Assay**

Production of nitrite was detected by a microplate colorimetric assay as described previously. Briefly, 100µl macrophage culture supernatant was combined with an equal volume of Griess reagent (1% sulfanilamide, 0.1% napthylethylene diamine dihydrochloride, 2.5% H₃PO₄) (Sigma Chemical Co.) and incubated at room temperature for 10 min. The OD₅₅₀ was read using an automated plate reader (VMAX, Molecular Devices Corp., Menlo Park, CA). Nitrite concentration was calculated from a NaNO₂ standard curve.

**Oxidative Burst Assay**

Induction of an oxidative burst was detected by a micro-fluorometric assay as previously described. Briefly, the macrophage lines and/or clones were collected, resuspended to $2 \times 10^5$/ml in DPBS supplemented with 2% HIFBS, and plated at 100 µl/well in a 96-well flat-bottom microtiter culture plate. Following the titration and addition of Zymosan A (Sigma Chemical Co.) or other triggering agent to the appropriate
wells, DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate, Molecular Probes) was added to all wells at a final concentration of 10μM and incubated at 37°C, 5% CO₂ for 60 min. The fluorescence intensity was determined at 485±10 nm excitation and 530±12.5 nm emission wavelengths using an automated fluorometer (FluoroCount, Packard Instrument Co, Inc., Meriden, CT) and the data expressed as relative fluorescent units (RFU).

**Cell-ELISA**

TNFα receptor expression on RAW 264.7 and C57Bl/6J spleen derived macrophages was measured using an ELISA modified for use on adherent cell populations. The cell cultures were washed three times with serum-free PBS and then fixed with 0.05% glutaraldehyde (Sigma Chemical Co.) in PBS at 4°C for 30 min. Following fixation, the cells were washed three times with saline and covered with DPBS containing 10% heat-inactivated horse serum (HS) and 5μg/ml human IgG (HGG). The DPBS/HS/HGG was then removed and replaced with 5 μg/ml of the appropriate primary antibody, anti-TNF-R1, anti-TNF-R2, or normal rabbit IgG, diluted in DPBS/HS/HGG. The cells were incubated for 60 min at room temperature and then rinsed three times with saline. The cells were then incubated for 30 min at room temperature with an optimal concentration of a peroxidase conjugated donkey anti-rabbit Ig (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted in DPBS/HS/HGG. Excess peroxidase conjugate was then removed by three washes with PBS containing 0.05% Tween 20 followed by three washed with PBS alone. Antibody binding to the cells was detected by incubating the cells with 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine (TMB, Sigma Chemical
Co.) and 0.05 M phosphate-citrate buffer containing 0.03% sodium perborate (Sigma Chemical Co.) for 30 min at room temperature in the dark. The supernatant was then transferred to another microwell plate containing 50 μl 2 M sulfuric acid and the OD₄₅₀ was read on an automated plate reader.

**FACS Analysis**

For surface phenotyping of the macrophage clones, the clones were resuspended in PBS containing 0.1% Azide, 2% HIFBS, and 25μg/ml Human IgG. They were then incubated with the following panel of antibodies: anti-CD4, anti-CD8, anti-ThB, anti-Mac-1, anti-F4/80, anti-CD40, anti-CD80, anti-CD86, or biotinylated anti-IA<sup>b</sup>d for 30 min at room temperature. The cells were rinsed with DPBS and incubated with either an FITC anti-rat or an FITC conjugated Neutralite Avidin for 30 min at room temperature in the dark. Following the incubation, the cells were washed with DPBS and analyzed on a FACStar Flow Cytometer (Becton Dickinson).

**RT-PCR Analysis**

Cells were plated at 1.2x10⁶/well in a 6-well plate (Falcon, Fisher Scientific). Following stimulation, the cells were treated with RNAzol (Gibco BRL) and the RNA extracted following the manufacturer’s instructions. The RNA was then reverse-transcribed into cDNA with MMLV-reverse transcriptase (Promega, Madison, WI). Two microliters of each cDNA sample were then used in a PCR reaction containing amplifier sets directed against G3PDH, IL-1β, TNFα, and i-NOS (Clontech, Palo Alto, CA). The
reactions were then subjected to an automated temperature cycling program consisting of
the following: Step 1: 94°C for 45 sec, Step 2: 60°C for 45 sec, Step 3: 72°C for 1 min,
Step 4: 72°C for 7 min, and Step 5: 4 °C hold. Steps 1-3 were repeated for 25 cycles for
G3PDH and IL-1β, 30 cycles for iNOS, and 20 cycles for TNFα. Then, 10μl of each
PCR product was loaded onto a 1.5% ethidium bromide containing agarose gel and
electrophoresed at ~100V for 45-60 min. The gel was then visualized and photographed
using Polaroid Type 665 Positive/Negative film (Fisher Scientific). The negatives were
then scanned for densitometric analysis using the MCID-M4 Image Analysis software
(Imaging Research, Inc., St. Catharines, Ontario). Relative induction for IL-1β, TNFα,
and iNOS was determined by first normalizing each gene with the values obtained for the
housekeeping gene, G3PDH. Values were then expressed as relative densitometric units.

Western Blot Analysis

Cells were plated at 3x10^5/well in 24-well tissue culture plates (Corning, Fisher
Scientific). Following stimulation, the cells were harvested in 50 μl boiling 2X
electrophoresis sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006%
bromphenol blue, 2% β-mercaptoethanol). Samples were boiled for an additional 5
minutes prior to protein separation by SDS-PAGE on 15% minigels. Gels were then
equilibrated in transfer buffer (48 mM Tris, pH 9.2, 39 mM glycine, 1.3 mM SDS, and
20% methanol) for 15 minutes prior to transfer. Protein transfer to BioBlot-NC
nitrocellulose membranes (Corning Costar Corp., Kennebunk, ME) was performed at 15V
for 30 min using a Trans-Blot® SD Semidry Electrophoretic Transfer Cell (BioRad,
Richmond, CA). Dried membranes were blocked with gentle agitation in PBS containing 0.1% Tween 20 and 1% BSA (blocking buffer) for 30 min at 37°C. The membranes were then probed using a polyclonal rabbit anti-iNOS at 1:10,000 (Transduction Laboratories, Lexington, KY) in blocking buffer for 30 min at 37°C, washed for 10 min x 2 washes in PBS containing 0.1% Tween-20 (PBS-T), followed by incubation with an HRP-conjugated donkey anti-rabbit IgG at 1:20,000 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in blocking buffer for an additional 30 min at 37°C, and then washed three times for 10 min each in PBS-T. Antibody bound proteins were detected using an ECL™ western blotting analysis system (Amersham Life Science, Arlington Heights, IL) and the membranes were exposed to Kodak BIOMAX ML X-ray film (Fisher Scientific). Membranes were then stripped (2% w/v SDS, 62.5 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol, 50°C for 30 min with occasional agitation), washed twice with PBS-T and placed in blocking buffer at 37°C for 30 min, and re-probed using a monoclonal mouse anti-β-actin at 1:5000 (Sigma) followed by a HRP-conjugated goat anti-mouse IgG at 1:40,000 (Jackson ImmunoResearch) to ensure loading of equivalent amounts of protein. The films were then scanned for densitometric analysis utilizing the UN-SCAN-IT™ gel automated digitizing system (Silk Scientific, Inc., Orem, UT) and the values expressed as relative densitometric units.
CHAPTER 3

ROLE OF TNFα RECEPTORS IN MACROPHAGE ACTIVATION

Effect of Murine Versus Human TNFα in the Induction of Macrophage Nitric Oxide Production

We and others have previously demonstrated that the induction of nitric oxide production requires synergistic signaling of the macrophages by both TNFα and IFNγ. To determine which TNFα receptor, TNF-R1 or TNF-R2, was the predominant receptor involved in the TNFα dependent activation of macrophage effector function, the specificity of hTNFα for TNF-R1 was exploited. Macrophages generated from spleen cell cultures were incubated with equivalent concentrations of either mTNFα or hTNFα in the presence of IFNγ (100 U/ml) for 48 hr after which the culture supernatants were assayed for the accumulation of nitrite. Human TNFα was approximately 12-fold less effective in inducing NO₂ in these primary cultures of macrophages, such that induction of 11 μM nitric oxide (~50% of maximal response observed) required 5 ng/ml hTNFα as compared to 0.4 ng/ml mTNFα (Figure 6A). In order to assess whether the activation state of the macrophages plays a role in the responsiveness to mTNFα versus hTNFα we compared the response patterns of C57Bl/6J spleen derived macrophages to RAW 264.7, a macrophage cell line, and to C57Bl/6J peritoneal elicited cells (PEC). Using 50% of the maximal response as a point for comparison, hTNFα was 5-fold less effective than mTNFα on RAW 264.7 (Figure 6B), 8-fold less effective than mTNFα on PEC (Figure 6C), and 12-fold less effective than mTNFα on spleen derived macrophages.
Figure 6. Effect of Murine TNFα vs. Human TNFα on the Induction of Macrophage Effector Function. Murine TNFα (closed circles) and Human TNFα (closed squares) were titrated onto (A) spleen-derived macrophages, (B) RAW 264.7, or (C) peritoneal elicited cells in the presence of 100 U/ml IFNγ and cultured for 48 hr. Following incubation, culture supernatants were assayed for nitrite accumulation. The dotted line represents IFNγ background. Data are presented as the arithmetic means of triplicate macrophage cultures ± SD and are representative of at least two separate experiments.
Relative Expression of TNF-R1 and TNF-R2 on RAW 264.7 and C57BL/6J Macrophages

To compare the relative expression of the two TNFα receptors on the macrophages used in this study cell-ELISAs were performed using purified anti-TNF-R1 and anti-TNF-R2 polyclonal antibodies. As depicted in Figure 7, the level of expression of TNF-R1 and TNF-R2 was approximately equal for RAW 264.7 (OD$_{450}$ 1.0 and 0.9, respectively). In contrast, spleen derived macrophages were found to have a significantly lower level of expression of TNF-R1 than TNF-R2 (OD$_{450}$ of 0.7 and 1.2, respectively).

After adjusting the OD$_{450}$ values by subtracting the normal rabbit IgG background, the ratio of TNF-R1 to TNF-R2 expression on RAW 264.7 was approximately 1.1, while the ratio for the spleen derived macrophages was 0.58.

Antagonistic Effect of Anti-TNF-Receptor Antibodies on Macrophages

In order to define the roles of TNF-R1 and TNF-R2 in the induction of macrophage effector function, anti-TNF-R1 and anti-TNF-R2 were initially titrated onto cultures of splenic macrophages in the presence of mTNFα and IFNγ. Anti-TNF-R1 reduced the ability of mTNFα to stimulate nitrite production in a dose-dependent fashion from 0.3-2.5 µg/ml (Figure 8A). Concentrations ≥ 2.5 µg/ml of anti-TNF-R1 reduced the level of NO$_2$ production induced by 3.9 ng/ml mTNFα to the background level of 3 µM which was obtained by stimulation with IFNγ alone. Macrophages incubated overnight in the presence of 5 µg/ml anti-TNF-R1 and stained for viability by trypan exclusion were found to be approximately 100% viable (data not shown), indicating that the anti-TNF-R1
Figure 7. Relative Expression of TNF-R1 and TNF-R2 on RAW 264.7 and C57BL/6J Macrophages. Each cell culture was incubated with 5 μg/ml of either anti-TNF-R1, anti-TNF-R2, or normal rabbit IgG followed by incubation with an optimal concentration of a peroxidase conjugated donkey anti-rabbit Ig. Data are presented as the arithmetic mean of at least 2 separate experiments ± SD, each experiment was run in either duplicate or triplicate OD_{450} measurements.
Figure 8. Titration of Anti-TNF-Receptor Antibodies on Macrophages. Anti-TNF-R1 (A) and anti-TNF-R2 (B) were titrated onto cultures of spleen-derived macrophages in the presence of 3.9 ng/ml mTNFα and 100 U/ml IFNγ and assessed for their ability to block the induction of nitric oxide following a 48 hr incubation period. Data are presented as the arithmetic means of triplicate macrophage cultures ± SD.
was inhibiting function rather than causing macrophage cell death. Addition of 2.5 μg/ml anti-TNF-R2 to macrophages in the presence of 3.9 ng/ml mTNFα resulted in a 40% reduction in the level of NO₂ production and this degree of inhibition could not be increased by increasing the concentration of anti-TNF-R2 (Figure 8B).

To determine if the inhibitory effect of anti-TNF receptor antibodies could be overcome by increasing concentrations of TNFα, macrophages generated from spleen cell cultures were incubated with rIFNγ and mTNFα in the presence of 5 μg/ml anti-TNF-R1 or 20 μg/ml anti-TNF-R2 for 48 hr after which the supernatants were assayed for nitrite accumulation (Figure 9A). Treatment with anti-TNF-R2 resulted in a partial inhibition of the mTNFα mediated induction of NO₂ production whereas incubation with anti-TNF-R1 completely inhibited NO₂ production. Increasing concentrations of mTNFα up to 12ng/ml (8,000 U/ml) could not supercede the blocking effect of anti-TNF-R2 (data up to 4 ng/ml are shown in Figure 9A). In contrast, when RAW 264.7 were subjected to the same experimental conditions as described above, increasing concentrations of mTNFα were able to overcome the inhibition by anti-TNF-R2 (Figure 9B). As was observed with primary cultures of macrophages, the addition of antibodies against TNF-R1 in the presence of mTNFα effectively blocked the production of nitric oxide.

Since TNF-R1 appeared to be the dominant receptor involved in the induction of macrophage effector function, several experimental approaches were attempted to determine whether ligation of TNF-R1 would induce production of NO₂. Incubation of RAW 264.7 with IFNγ and either 5 μg/ml anti-TNF-R1 followed by F(ab)’₂ goat
Figure 9. Antagonistic Effect of Anti-TNF-Receptor Antibodies on Macrophages.

Murine TNFα was titrated onto cultures of (A) spleen-derived macrophages or (B) RAW 264.7 and incubated with 100 U/ml IFNγ alone (closed circles), in the presence of 5 μg/ml anti-R1 (closed squares), or in the presence of 20 μg/ml anti-R2 (closed triangles) for a period of 48 hr after which the supernatants were assayed for nitrite accumulation. The dotted line represents IFNγ background. Data are presented as the arithmetic means of triplicate macrophage cultures ± SD and are representative of at least two separate experiments.
anti-rabbit Ig or with anti-TNF-R1 immobilized on plastic did not induce significant nitric oxide production (data not shown).

**Effect of IFNγ and GM-CSF Pretreatment on the Induction of Macrophage Effector Function**

One possible explanation for the difference in response pattern between spleen derived macrophages and RAW 264.7 was the state of activation of the cells prior to exposure to TNFα. To investigate this hypothesis both spleen derived macrophages and RAW 264.7 were preincubated with either 100 U/ml IFNγ, 400 U/ml GM-CSF, or media alone for 48 hours followed by stimulation with either mTNFα or hTNFα as previously described. Treatment with either IFNγ or GM-CSF was capable of heightening the responsiveness of primary macrophages to both mTNFα and hTNFα (Figure 10A,B,C). IFNγ pretreatment resulted in a 40% and 170% increase in the maximal induction of NO₂ production in response to mTNFα and hTNFα, respectively (Figure 10B). Pretreatment with GM-CSF yielded a similar result such that the maximal induction of NO₂ production in response to mTNFα and hTNFα were increased by 35% and 120%, respectively (Figure 10C). However, even with IFNγ or GM-CSF priming, hTNFα was about 3-fold less effective than mTNFα in inducing nitric oxide in primary macrophages, which was nonetheless an improvement over the 12-fold difference seen with non-primed macrophages. Moreover, regardless of pretreatment, mTNFα yielded nitrite levels that were 20-40% in excess of those achievable with the superphysiologic concentration of 20 ng/ml hTNFα.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 10. Effect of Cytokine Priming on the TNFα Mediated Induction of Macrophage Effector Function. Spleen-derived macrophages were preincubated with either media alone (A), 100 U/ml IFNγ (B), or 400 U/ml GM-CSF (C) for 48 hours followed by stimulation with either mTNFα (closed circles) or hTNFα (closed squares) in the presence of 100 U/ml IFNγ for an additional 48 hours. Following incubation, culture supernatants were assayed for nitrite accumulation. The dotted line represents IFNγ background. Data are presented as the arithmetic means of triplicate macrophage cultures ± SD and are representative of at least two separate experiments.
Unlike the spleen-derived macrophages, pretreatment of RAW 264.7 with IFNγ or GM-CSF did not enhance the maximal responses elicited by either mTNFα or hTNFα (Figure 11A,B,C). IFNγ pretreatment slightly downregulated the responsiveness of RAW 264.7 14% and 23% for mTNFα and hTNFα, respectively (Figure 11B). Also in contrast to resting macrophage cultures, stimulation of RAW 264.7 with hTNFα, albeit at higher doses, was capable of eliciting levels of nitric oxide equivalent to those obtained by stimulation with mTNFα.

To determine if the effects noted in Figure 10 were due to modulation of surface expression of the two TNFα receptors, cell ELISAs were performed on spleen-derived macrophages which had been preincubated with IFNγ, GM-CSF, or media alone for 48 hr. Pretreatment of macrophages with either IFNγ or GM-CSF had little effect on the relative expression of either TNF-R1 and TNF-R2 or on the ratio of TNF-R1 to TNF-R2 expression (data not shown).
Figure 11. Effect of Cytokine Priming on the TNFα Mediated Induction of Effector Function in RAW 264.7. RAW 264.7 were preincubated with either media alone (A), 100 U/ml IFNγ (B), or 400 U/ml GM-CSF (C) for 48 hours followed by stimulation with either mTNFα (closed circles) or hTNFα (closed squares) in the presence of 100 U/ml IFNγ for an additional 48 hours. Following incubation, culture supernatants were assayed for nitrite accumulation. The dotted line represents IFNγ background. Data are presented as the arithmetic means of triplicate.
CHAPTER 4
LPS STIMULATION OF TNFα-RECEPTOR DEFICIENT MACROPHAGES

Generation and Characterization of Wild-Type and TNF-Receptor Negative Macrophage Lines

Immortalized macrophage cell lines were established by infecting the bone marrow of TNFα-receptor negative (TRN) C57BL/6J mice and from wild type C57BL/6J mice (B6/J2) with the murine recombinant J2 retrovirus containing the v-myc and v-raf oncogenes as previously described. The immortalized cell lines were subsequently cloned by limiting dilution and characterized for surface phenotype and macrophage effector functions. The B6/J2 (Figure 12A-D) and TRN (Figure 12E-H) cell lines were analyzed for the expression of surface molecules characteristic of macrophages. Both B6/J2 and TRN were found to express the macrophage markers F4/80, Mac-1 and CD14, but did not express T (CD4, CD8) or B (ThB) cell markers (Figure 12 panels A, B, E and F). The immortalized lines were also assayed for expression of accessory/costimulatory molecules: Ia, CD40, CD80, and CD86 (Figure 12 panels C, D, G and H). The B6/J2 and TRN both expressed high levels of CD86 and moderate levels of Ia, CD40, and CD80.

The production of reactive oxygen and reactive nitrogen intermediates are two hallmark microbicidal effector mechanisms employed by macrophages/monocytes. Therefore, the cell lines and clones were characterized for their ability to generate oxygen and nitrogen intermediates upon stimulation. The oxidative burst responses induced by
Figure 12. Surface Phenotyping of Immortalized Macrophage Lines. Each of the B6/J2 (panel A-D) and TRN (panel E-H) macrophage clones were analyzed by flow cytometry. Approximately 1x10^6 cells were stained with a panel of antibodies reactive with the following determinants and compared to unstained controls: A and E, F4/80, Mac-1, CD4, CD8, and ThB; B and F, CD14; C and G, Ia^b; D and H, CD40, CD80, CD86. Histogram profiles of 10,000 cells from a representative clone for both B6/J2 and TRN are displayed.
zymosan in the macrophage lines and clones were compared with those induced in RAW 264.7 and GM-CSF primed or unprimed spleen-derived macrophages. The oxidative response of the B6/J2 line was nearly identical to that of GM-CSF primed spleen-derived macrophages (Figure 13A) and was somewhat higher than the response of RAW 264.7 (Figure 13B). The three wild-type clones tested (B6/J2.1, B6/J2.2, B6/J2.3) all responded with an intensity similar to the parent line (Figure 13B). Similarly, all the TNFα-receptor negative clones responded as well, or nearly as well, as the most responsive wild-type clone (B6/J2.1) (Figure 13C).

All of the clones could be induced to produce nitric oxide upon stimulation with LPS plus IFNγ (Figure 14). The B6/J2 line responded well to either LPS or TNFα, in the presence of IFNγ, with a minimal response to either LPS or IFNγ alone (Figure 14A). Each of the wild-type clones responded in a similar fashion to the parent line (Fig 14B). The TNFα-receptor negative clones also produced nitric oxide in response to LPS plus IFNγ, although there was variability in their responses to IFNγ alone (Figure 14C). As expected, the TRN clones were completely unresponsive to stimulation by TNFα plus IFNγ (Figure 14D), while the B6/J2 responded in a dose-dependent manner. Immortalized macrophage lines deficient in either TNF-R1 (DC-1) or TNF-R2 (AC-2) were also characterized and found to be phenotypically and functionally indistinguishable from the other macrophage lines (data not shown).
Figure 13. Assessment of Functional Characteristics via Induction of Oxidative Burst.

The oxidative capacity of the B6/J2 macrophage line and clones was measured and compared to (A) spleen derived C57Bl6/J macrophages and (B) RAW 264.7. (C) The TRN macrophage clones were compared to a representative B6/J2 clone. Oxidative burst was induced and measured as described in Materials and Methods. The data, representative of at least two separate experiments, are presented as the arithmetic means of triplicate macrophage cultures ± SD and expressed as Relative Fluorescent Units.
Figure 14. Assessment of Functional Characteristics via Induction of Nitric Oxide Production. (A) Comparison of the B6/J2 parental line and RAW 264.7. (B) B6/J2 clones compared to the parent B6/J2 line. (C) TRN clones compared to a representative B6/J2 clone. The cells were stimulated with media alone, 10U/ml IFNγ, 100 ng/ml LPS, LPS + IFNγ, or 5 ng/ml mTNFα + IFNγ, as indicated, and cultured for 48h. (D) Titration of mTNFα onto cultures of TRN clones and a representative B6/J2 clone in the presence of 10 U/ml IFNγ and cultured for 48h. Following incubation, culture supernatants were assayed for nitrite accumulation. Data, representative of at least two separate experiments, are presented as the arithmetic means of triplicate macrophage cultures ± SD (with IFNγ background subtracted out in D).
Involvement of the TNF\(\alpha\) Autocrine Loop in the Induction of Macrophage NO Production Under Serum-Free Conditions

The ability of the TRN to respond to low concentrations of LPS in the presence of serum indicated that autocrine stimulation by TNF\(\alpha\) was not critical for CD14/LBP-mediated activation of nitric oxide generation. To determine if autocrine stimulation by TNF\(\alpha\) was critical for LBP-independent activation of macrophages by LPS, B6/J2 and TRN were stimulated with up to 10 \(\mu\text{g/ml}\) LPS and 10 U/ml IFN\(\gamma\) in serum-free media. LPS stimulation of the B6/J2 in both the presence and absence of serum resulted in the production of NO although a higher concentration of LPS was required for optimal stimulation in the absence of serum (Figure 15). Conversely, stimulation of the TRN in the absence of serum resulted in a very weak nitric oxide response that was \(\leq 50\%\) of the response of TRN in the presence of serum and of the response of B6/J2 in the absence of serum (Figure 15).

Given the poor NO production by TRN in response to LPS in the absence of serum, it was important to determine if other LPS-mediated responses of TRN were equally affected. Hence, RNA harvested from both B6/J2 and TRN following a 4-hr stimulation with 0-10 \(\mu\text{g/ml}\) LPS under serum-free conditions was analyzed via RT-PCR for the expression of iNOS, IL-1\(\beta\), and TNF\(\alpha\) (Figure 16). The PCR product bands were then scanned and analyzed by densitometry (Figure 17A-D). LPS stimulation resulted in a dose-dependent induction of IL-1\(\beta\), TNF\(\alpha\), and iNOS for both B6/J2 and TRN. It is of interest that under serum-free conditions, LPS stimulation induced a higher level of iNOS RNA expression in TRN than in B6/J2 (Figure 17D). Supernatants from cultures treated...
Figure 15. Involvement of TNFα in the Induction of Nitric Oxide Production Under Serum-Free Conditions. LPS was titrated onto cultures of B6/J2 (circles) and TRN (triangles) in the presence (closed symbols) and absence of serum (open symbols), cultured for 48h, and assayed for nitrite accumulation. Data are presented as the arithmetic means of triplicate macrophage cultures ± SD.
Figure 16. LPS Induced Gene Expression in Serum-Free Media I. RT-PCR was performed on RNA extracted from macrophages cultured in the absence of serum and treated with media alone or the indicated concentration of LPS for 4h. Upper lanes 1-8 contain G3PDH PCR products, upper lanes 10-17 contain IL-1β PCR products, lower lanes 1-8 contain TNFα PCR products, and lower lanes 10-17 contain iNOS PCR products. Data is representative of at least two individual RNA isolations.
Figure 17. LPS Induced Gene Expression in Serum-Free Media II. (A-D) Densitometric analysis was performed on PCR products of B6/J2 (open bars) and TRN (closed bars) presented in Figure 16. The data was normalized for G3PDH and expressed as Relative Densitometric Units.
identically as those used for RT-PCR were collected and assayed for biologically active TNFα (Figure 18). LPS induced TNFα production in both B6/J2 and TRN in a dose-dependent manner, as indicated by the percent cytotoxicity of L929, with 10 μg/ml LPS resulting in an equivalent production of TNFα by the two macrophage lines.

Figure 18. Induction of TNFα Production Under Serum-Free Conditions. LPS was titrated onto cultures of B6/J2 (open bars) and TRN (hatched bars) in serum-free media for 4h. Following incubation, the supernatants were assayed for TNFα bioactivity utilizing the L929 bioassay. Data, representative of at least two separate experiments, are presented as the arithmetic means of triplicate macrophage cultures.
In an attempt to reconcile the relatively high iNOS mRNA expression with the low production of NO, lysates from TRN and B6/J2, stimulated with 10 μg/ml LPS under serum-free conditions for 0-48 hrs, were analyzed for the presence of iNOS protein (Figure 19). Stimulation of both B6/J2 and TRN resulted in a time-dependent increase in the amount of iNOS protein detected, regardless of the presence (Figure 19B) or absence (Figure 19A) of IFNγ. Densitometric analysis revealed that stimulation of TRN in the presence of IFNγ for 12 hr resulted in ~60% less iNOS protein as compared to B6/J2 with the difference decreasing to 20% by 48 hr (Figure 19D). In the absence of IFNγ, LPS stimulation of TRN consistently resulted in 80-90% less iNOS protein production compared to B6/J2 (Figure 19C).

To confirm the critical role of autocrine TNFα in LBP-independent mechanisms of macrophage activation, the B6/J2 were stimulated under serum-free conditions in the presence of increasing concentrations of the soluble murine TNFα-receptor type 1. As shown in Figure 20, soluble TNFα inhibitor reduced the amount of NO generated with 10 μg/ml soluble TNFα-R1 effecting a 50% decrease in the nitric oxide response induced by 1 μg/ml LPS and 10 U/ml IFNγ.
Figure 19. LPS Induced iNOS Protein Expression Under Serum-Free Conditions. B6/J2 and TRN were stimulated for 0-48 hr with 10 μg/ml LPS alone (A) or in the presence of IFNγ (B). Cell lysates were electrophoresed and subjected to western blot analysis. Data presented are representative of at least two individual experiments. C and D, Densitometric analysis of the gels presented above.
Figure 20. Soluble Murine TNF-R1 Inhibition of B6/J2 Nitric Oxide Production. B6/J2 macrophages were stimulated in serum-free media (close diamonds) with either 6 U/ml IFN\(\gamma\) (closed triangle), 1 \(\mu\)g/ml LPS (closed square), or LPS + IFN\(\gamma\) in the presence of increasing concentrations of soluble murine TNF-R1 (closed circle) for 48 h. Following incubation, culture supernatants were assayed for nitrite accumulation. Data, representative of at least two separate experiments, are presented as the arithmetic means of triplicate macrophage cultures ± SD.
The induction of macrophage effector function is, in part, a result of synergy between LPS and IFNγ. LPS alone is capable of inducing the production and secretion of TNFα while IFNγ not only enhances TNFα production, but also upregulates the surface expression of TNFα receptors allowing for the formation of a TNFα autocrine loop. TNFα, in the presence of IFNγ, is capable of providing the necessary signals to induce the production of nitric oxide, a classic macrophage microbicidal and tumoricidal effector molecule. The experiments described in this dissertation were designed not only to determine through which of the TNFα receptors the effects of TNFα are mediated, but also to assess the role of autocrine TNFα in the induction of macrophage effector function.

As demonstrated herein, the primary TNFα-mediated signals involved in the activation of macrophage effector function are transduced through TNF-R1 insofar as 1) stimulation via TNF-R1 with hTNFα is sufficient for the induction of nitric oxide generation, and 2) addition of anti-TNF-R1 antibodies completely inhibits nitric oxide production. Although TNF-R1 is critical for the TNFα-mediated induction of macrophage effector function, signaling through TNF-R2 contributes to the intensity of the macrophage response such that addition of anti-TNF-R2 partially inhibited macrophage activation.
The cloning of the two murine TNFα receptors demonstrated that although TNF-R1 had a similar affinity for both recombinant mTNFα and hTNFα, TNF-R2 showed affinity for only mTNFα, thus defining TNF-R2 as a species-specific receptor. The ability of hTNFα to bind to TNF-R1 but not to TNF-R2 provides a model allowing separation of TNF-R1 mediated effects from TNF-R2 effects. Using this model, TNF-R1 was found to mediate most of the biological effects of TNFα including initiation of cellular cytotoxicity and apoptosis, enhancement of oxidative burst, as well as mediation of systemic shock during sepsis. Our finding that hTNFα is effective in the IFNγ-dependent induction of nitric oxide in both cultured macrophages and a macrophage cell line indicates that ligation of TNF-R1, by itself, is sufficient for TNFα-mediated induction of macrophage effector function. Furthermore, the ability of anti-TNF-R1 to abrogate the induction of a nitric oxide response by murine TNFα indicates that ligation of TNF-R2, by itself, is not sufficient for TNFα mediated signaling of macrophage activation. These observations on TNFα mediated signaling of mature macrophages conforms to the current body of evidence that TNF-R1 is the dominant receptor in TNFα mediated events.

In contrast to the abrogating effect of anti-TNF-R1 antibodies, treatment of macrophages with anti-TNF-R2 effected a partial inhibition of TNFα mediated signaling. The TNFα mediated generation of nitric oxide in RAW 264.7 appears to be dependent on TNF-R2 only at low doses of TNFα, such that 1) increasing concentrations of mTNFα overcome the partial inhibition elicited by antibody blocking of TNF-R2, and 2) stimulation of RAW 264.7 with high doses of hTNFα induces responses as intense as
stimulation with mTNFα. These observations are consistent with the observed effects of hTNFα and anti-TNF-R2 on L929 fibroblasts. Based on the observations that hTNFα could effectively induce cellular cytotoxicity, albeit at higher concentrations than required for mTNFα, Tartaglia et al. proposed a "ligand passing" role for TNF-R2. This model suggests that TNF-R2 plays a role only at low TNFα concentrations and is based on the hypothesis that the higher affinity TNF-R2 rapidly associates and dissociates with TNFα, increasing the local concentration of TNFα at the cell surface which results in a more rapid association of TNFα with TNF-R1. However, data have been accumulating recently that indicate the role of TNF-R2 may not be so simple.

The antagonistic effect of anti-TNF-R2 on spleen derived macrophages was quite different from that seen with RAW 264.7. Whereas RAW 264.7 was only dependent on TNF-R2 at low TNFα concentrations, splenic macrophages exhibited a dependency on TNF-R2 over all concentrations of TNFα tested. The partial inhibition of mTNFα mediated macrophage activation by anti-TNF-R2 could be overcome in RAW 264.7 by increasing the mTNFα concentration, but was unaffected by increasing concentrations of mTNFα in splenic macrophages. This difference is also observed by comparing the differential response patterns elicited by mTNFα and hTNFα on the spleen derived macrophages (Fig. 6A) with the response patterns elicited by TNFα on RAW 264.7 (Fig. 6B) and PEC (Fig. 6C). In cultures of RAW 264.7 and PEC, high doses of hTNFα can induce concentrations of nitric oxide equivalent to those induced by mTNFα, while in cultures of splenic macrophages, increasing doses of hTNFα are not able to achieve the level of nitric oxide generation elicited by mTNFα. Together these results suggest that the
dependency on TNF-R2 in spleen-derived macrophages is greater than in RAW 264.7 or PEC.

One key difference between either RAW 264.7 or PEC and spleen-derived macrophages that may account for the varying dependence on TNF-R2 is their functional state. RAW 264.7, J774A.1, and elicited macrophages (as opposed to resident macrophages) have been shown to be in a "primed" state such that they constitutively produce cytokines such as IFNβ, release significant quantities of prostaglandins, and can be induced to express significant effector function by LPS without exogenous IFNγ⁴. In contrast, cultured macrophages appear to be more down-regulated or "resting" in that they do not produce nitric oxide in response to LPS alone and do not express cytokine mRNA or protein without stimulation⁴,²⁶,⁹²,¹⁰⁵. It is well established that responsiveness of macrophages to a given stimulus depends on their state of activation⁴,¹⁰⁶. It is therefore conceivable that the dependence on TNF-R2 in the TNFα mediated signaling of macrophage effector function may be related to the functional state of the macrophage. This current line of thought is supported by our results which demonstrate that cytokine priming of spleen-derived macrophages decreased their dependence on TNF-R2 as compared to non-primed macrophages.

The results of Vandenabeele et al.¹⁰⁷ on T cell apoptosis and our results on macrophage nitric oxide generation suggest a model for TNFα stimulated events which involves two distinct signals which can be mediated by each of the two TNFα receptors. Through triggering by agonistic mAbs and receptor-specific mutated proteins, Vandenabeele et al. demonstrated that the role of TNF-R2 in PC60 cells was not that of a
ligand passer, but instead, was that of an active participant in the intracellular signaling pathway leading to apoptosis. Consequently, the TNFα-mediated induction of NO₂ in cultured macrophages may involve a signal delivered by TNF-R2 in spleen-derived macrophages which amplifies (or synergizes) with the signal transduced via TNF-R1, culminating in the activation of nitric oxide synthase. While cytokine priming of splenic macrophages was capable of enhancing the maximal induction of nitric oxide production in response to both species of TNFα and increasing the autonomy of TNF-R1, priming failed to enhance expression of either TNF-R1 or TNF-R2. This suggests that GM-CSF or IFNγ can initiate signaling pathways which either bypass signaling via TNF-R2 or provide signals similar to those mediated through TNF-R2.

The mechanism by which TNF-R1 and TNF-R2 signal the induction of nitric oxide synthase is still unclear. Both VanArsdale and Ware and Darnay et al. have demonstrated that a serine/threonine kinase associates with the cytoplasmic region of TNF-R1 and is subsequently activated by TNFα binding. Rothe et al. have identified a group of putative signal transducers associated with the cytoplasmic domain of the hTNF-R2 termed TNFα receptor-associated factor 1 (TRAF1) and TRAF2. It has also been shown that maximal expression of the macrophage nitric oxide synthase is dependent upon two discrete regulatory regions upstream from the putative TATA box. Region I contains LPS-related response elements, including NF-IL6 and a binding site for NF-κB, while region II contains motifs for binding IFN-related transcription factors. Whether both TNF-R1 and TNF-R2 activate transcription factors which may bind to region I has yet to be determined.
The second aim of this dissertation was to better understand the contribution of autocrine TNFα in the induction of macrophage effector function. As demonstrated herein, TNFα autocrine stimulation was critical for the LPS-mediated induction of macrophage NO production via the LBP-independent pathway insofar as 1) the NO response of macrophages which lack both types of TNFα receptors was significantly impaired when stimulated with LPS in the absence of serum whereas wild-type macrophages were unaffected (Fig 15) and 2) LBP-independent LPS stimulation of wild-type macrophages in the presence of a soluble TNFα inhibitor resulted in a dose-dependent reduction in nitric oxide generation (Fig 19).

The macrophage lines developed for use in this study were generated via selective immortalization with retroviral vectors carrying the \textit{v}-\textit{myc} and \textit{v}-\textit{raf} oncogenes \textsuperscript{93,100}. The immortalized lines/clones were phenotypically and functionally indistinguishable from either RAW 264.7 or primary spleen-derived macrophages. Not only did the immortalized lines and clones express the specific macrophage markers (Mac-1, F4/80, CD14) but they also displayed characteristic accessory markers (Ia, CD40, CD80, CD86) providing an excellent model for studying macrophage interactions. Additionally, both wild-type B6/J2 and the TNF-receptor deficient TRN were capable of producing NO in response to LPS and of generating an oxidative burst in response to stimulation with zymosan in a manner similar to that of RAW 264.7 and splenic macrophages.

CD14 has been well characterized as a receptor for LPS \textsuperscript{40,110,111}. CD14 mediated macrophage activation is a serum-dependent event, in that the circulating glycoprotein LBP facilitates the binding of LPS by CD14 \textsuperscript{112}. Furthermore, antibodies directed against
CD14 could only inhibit the response of a human monocytic cell line to LPS in the presence of serum. As shown by the current study, induction of macrophage NO production in the presence of serum requires 100-fold less LPS than that needed for similar production of NO in the absence of serum. Thus, the serum-independent, LBP-independent pathway appears to involve a low affinity receptor, since higher concentrations of LPS (≥ 1 μg/ml) were required to elicit a response.

Studies on CD14-knockout mice have elucidated the CD14-independent mechanisms of LPS activation and have revealed differential activation of macrophage functions by CD14-dependent and CD14-independent pathways. Perera et al. recently characterized a CD14-independent pathway of LPS activation which resulted in the production of TNFα and IL-1β with minimal induction of interferon-inducible protein (IP-10), whereas stimulation via CD14 resulted in the induction of all three. Consistent with results of Perera et al., the current study shows that LBP-independent LPS activation results in the induction of IL-1β, TNFα, and iNOS gene expression in both wild-type and TNF-receptor deficient macrophages. The dose-dependent induction of these genes corresponded to the significant production of end product with the exception of NO production by TRN. Although the LBP-independent LPS stimulation of TRN induced equivalent if not greater levels of mRNA for iNOS as compared to B6/J2, the amount of iNOS protein produced and NO released was markedly reduced. Taken together, these data suggest autocrine TNFα plays a critical role in post-transcriptional regulation of NO production by macrophages stimulated via LBP-independent pathways.
Previous studies have shown that iNOS is highly subject to post-transcriptional regulation\textsuperscript{112,115-117}. Vodovotz et al.\textsuperscript{115} linked TGF-β to the suppression of iNOS expression through enhanced degradation of iNOS mRNA, impaired translation, and accelerated protein degradation. Our data cannot be explained by autocrine TGF-β control of iNOS since the levels of TGF-β, as detected by ELISA, were significantly lower in TRN as compared to B6/J2 following stimulation with 10 µg/ml LPS under serum-free conditions either with or without IFNγ for 48 hr (data not shown). A second, more likely scenario was suggested by the results of Walker et al.\textsuperscript{117} in which increased iNOS degradation was ascribed to the proteolytic action of the cysteine protease calpain. Applying their findings to our own observations it is conceivable that the LPS-mediated induction of macrophage nitric oxide production in the absence of LBP requires the activation of a calpain inhibitor that appears to be autocrine TNFα-dependent. This scenario would thus provide a plausible explanation for the lower levels of iNOS protein detected in the LBP-independent LPS stimulation of TRN as compared to B6/J2.

The differential signaling of macrophage function observed in CD14 -/- macrophages\textsuperscript{114} and in TRN macrophages is intriguing. One explanation is that, in both cases, LPS is binding to, and signaling via, an as yet unidentified low affinity LPS receptor that initiates a signal cascade similar, but not identical, to that initiated through the CD14-associated signaling complex\textsuperscript{118}. An alternative explanation is suggested by the recent report that the CD14-associated signaling molecule(s) is a member of the Toll-like receptor (TLR) family of which 5 homologues have been identified\textsuperscript{119}. Binding of LPS to TLR-2 evidently requires LBP facilitation and appears to be slightly enhanced by CD14.
Although it is not yet clear if LPS or LBP/LPS complexes associate with the other members of the TLR family, the binding of different TLR proteins by LPS/LBP and CD14 would offer an explanation for the observed differential loss of signaling under either CD14-independent or LBP-independent conditions.

In summary, this dissertation has 1) characterized the roles of the two TNFα receptors in the TNFα-mediated activation of macrophages and 2) described a critical role for autocrine TNFα in serum-independent LPS-induced macrophage activation. We have provided direct evidence indicating that TNFα-mediated macrophage activation primarily utilizes TNF-R1 for the induction of nitric oxide. In addition, we have also demonstrated that the role of TNF-R2 is not limited to sequestration and facilitation of TNFα for TNF-R1 signaling, but rather appears to be an active participant in the induction of nitric oxide. Secondly, we have identified a critical role for autocrine TNFα in the post-transcriptional regulation of nitric oxide synthase in macrophages stimulated with LPS in the absence of serum. This would suggest that following stimulation with LPS in the absence of LBP, autocrine TNFα-mediated initiation of some unidentified signaling pathway is necessary for optimal iNOS activity. The mechanism of TNFα-mediated post-transcriptional regulation of iNOS has yet to be elucidated.
REFERENCES


32. Armitage RJ, Macduff BM, Spriggs MK, Fanslow WC. Human B cell proliferation and Ig secretion induced by recombinant CD40 ligand are modulated by soluble cytokines. J Immunol 1993 May 1;150(9):3671-80.


64. Ranheim EA, Kipps TJ. Tumor necrosis factor-alpha facilitates induction of CD80 (B7-1) and CD54 on human B cells by activated T cells: complex regulation by IL-4, IL-10, and CD40L. Cell Immunol 1995 Apr 1;161(2):226-35.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


111. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein [see comments]. Science 1990 Sep 21;249(4975):1431-3.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


VITA

ANNETTE RENE' CLEMONS-MILLER

Personal Data:  Date of Birth: August 15, 1968
Place of Birth: Fort Collins, Colorado
Marital Status: Married

Education:  Appalachian State University, Boone, North Carolina;
            Biology, B.S., December 1990
Appalachian State University, Boone, North Carolina;
            Biology, M.S., August 1993
East Tennessee State University, Johnson City, Tennessee;
            Microbiology and Immunology, PhD., anticipated 1998

Publications:  Miller, A.R. "Effect of high- versus moderate-intensity exercise on
Nieman, D.C., Miller, A.R., Henson, D.A., Warren, B.J.,
Gusewitch, G., Johnson, R.L., Davis, J.M., Butterworth, D.E.,
Nehlsen-Cannarella, S.L., "Effects of high- vs moderate-intensity
exercise on natural killer cell activity", Medicine in Science Sports
Nieman, D.C., Miller, A.R., Henson, D.A., Warren, B.J.,
Gusewitch, G., Johnson, R.L., Davis, J.M., Butterworth, D.E.,
Herring, J.L., Nehlsen-Cannarella, S.L., "Effect of high- vs
moderate-intensity exercise on lymphocyte subpopulations and
proliferative response", International Journal of Sports Medicine,
Stout, R.D., Li, Y., Miller, A.R., Lambe, D.W. Jr., "Staphylococcal
glycocalyx activates macrophage prostaglandin E2 and interleukin 1
production and modulates tumor necrosis factor alpha and nitric
4160-6.
Miller, A.R., Suttles, J., and Stout, R.D., "Cytokine priming
reduces dependence on TNF-R2 for TNF-mediated induction of

