2009

The Role of Myeloid-Derived Suppressor Cells in the Immunotherapy of Breast Carcinomas

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Dedication

This work is dedicated to my father, the late Dr. William B. Keeler, Jr., who has been an incredible and positive influence on my life. Through him I have learned the value of a hard day's work and have come to appreciate the significance of honesty and integrity above all else. The emphasis my dad put on family time and values has, in the past several years, come to be fully appreciated. It is unfortunate that these things are usually realized in hindsight and with increasing age and is also unfortunate that he is not here to witness this accomplishment. This degree and my previous bachelor’s degree have been made possible through his support of our family, and his belief in education and improving oneself by not accepting anything less than your best effort. I have learned to make my own way in life and to count on no one. I have learned to save for the future, for life is uncertain, but I have also learned that this uncertainty brings with it a requirement for living each day to its fullest and realizing what is really important in life, because life, in one moment can be changed forever or taken away. My goal, therefore, is to live my life in such a way as to leave me with no regrets, a goal that my father was able to accomplish and, I believe, took some solace in the knowledge that he had always done his best in his life and for his family. These past four years, therefore are in his memory, which shall never be forgotten.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>xv</td>
</tr>
<tr>
<td>Table of anti-cancer drugs</td>
<td>xx</td>
</tr>
<tr>
<td>Abstract</td>
<td>xxii</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>I. The ErbB Family of Receptor Tyrosine Kinases</td>
<td>1</td>
</tr>
<tr>
<td>A. Structure and Function</td>
<td>1</td>
</tr>
<tr>
<td>B. Signaling Pathways</td>
<td>6</td>
</tr>
<tr>
<td>II. Cancer Therapeutics</td>
<td>10</td>
</tr>
<tr>
<td>A. Chemotherapies</td>
<td>10</td>
</tr>
<tr>
<td>B. Monoclonal Antibodies</td>
<td>12</td>
</tr>
<tr>
<td>C. Tyrosine Kinase Inhibitors</td>
<td>15</td>
</tr>
<tr>
<td>D. Vaccines</td>
<td>17</td>
</tr>
<tr>
<td>E. Adoptive Immunotherapy</td>
<td>21</td>
</tr>
<tr>
<td>III. Suppressor Cells Abrogate Anti-Tumor Immune Responses</td>
<td>25</td>
</tr>
<tr>
<td>A. Regulatory T cells</td>
<td>25</td>
</tr>
<tr>
<td>B. Myeloid-Derived Suppressor Cells</td>
<td>27</td>
</tr>
<tr>
<td>i. Mechanisms of T cell Suppression by MDSC</td>
<td>28</td>
</tr>
<tr>
<td>ii. MDSC and COX-2</td>
<td>31</td>
</tr>
<tr>
<td>iii. Mechanisms of MDSC Induction</td>
<td>33</td>
</tr>
<tr>
<td>iv. MDSC and Soluble Factors</td>
<td>36</td>
</tr>
<tr>
<td>v. Subsets of MDSC and the Ly6G Molecule</td>
<td>37</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>40</td>
</tr>
</tbody>
</table>
Chapter 1 ..................................................................................................................52

Study Rationale ........................................................................................................52

FVBN202 Mice Bearing HER2/neu Positive MMC Tumors
Accumulate Large Numbers of CD11b+Gr1+ Cells in their
Spleens, Bone Marrow, and Blood ........................................................................54

Bryostatin-1/ Ionomycin Stimulation Followed by IL-2
Expansion Generates Highly Activated Neu-Specific
Effector T cells .......................................................................................................65

In Vivo Depletion of MDSC is Required for the Efficacy
of IL-2 Expanded Adoptively Transferred T cells ............................................72

The Efficacy of in vivo MDSC Depletion can be Directly
Correlated with the Effectiveness of the Anti-Tumor Response .................78

T Cell Expansion Using Alternating Gamma Chain
Cytokines Generates More Potent Anti-Tumor T cells
with Greater Levels of Expansion ......................................................................90

Adoptive Transfer of T cell Subsets Expanded Ex Vivo
with Alternating Gamma Chain Cytokines Inhibits
Tumor Growth when Combined with the Depletion
of MDSC in vivo .................................................................................................104

Gemcitabine is an Effective Inhibitor of MDSC in
Tumor Bearing FVBN202 Mice and Allows for the
Complete Rejection of HER2/neu Positive Mammary Tumors ....................120

Conclusions and Significance .............................................................................142

Chapter 2 ..................................................................................................................144

Study Rationale ....................................................................................................144

MDSC in Tumor-Bearing FVBN202 Mice Suppress
anti-CD3/CD28 Mediated Proliferation of both
CD4+ and CD8+ T cells ....................................................................................145
CD11b+Ly6G-Ly6C+ MDSC are Suppressive, While CD11b+Ly6G+Ly6C+ Cells are Not ........................................................148

Suppressive MDSC require Direct Cell-to-Cell Contact that is Independent of LFA-1/ICAM and PD-1/PD-1L Interactions to Inhibit the Proliferation of CD4+ and CD8+ T cells ................................................................. 151

Conclusions and Significance .........................................................................................170

Chapter 3 ..........................................................................................................................171

Study Rationale ...............................................................................................................171

MMC-Derived Soluble Factors Cause the Generation of MDSC .................................172

GM-CSF is Responsible for the Generation and Maintenance of MDSC.......................209

FVBN202 Mice Display Unique Subsets of MDSC with GM-CSF Selectively Driving the Generation of the Suppressive Ly6G-Ly6C+ Subset ................................................................. 221

Conclusions and Significance .........................................................................................235

Discussion ........................................................................................................................237

Chapter 1 ..........................................................................................................................237

Neu+ Tumors in FVBN202 Mice cause an Increase in MDSC that Prevents Successful Adoptive Immunotherapy ................................................................. 237

T Cell Expansion Using Alternating Gamma Chain Cytokines Results in Greater Expansion, Cytotoxicity, and Viability ............................................................................. 239

AIT Using T Cells Expanded in Alternating Gamma Chain Cytokines is Only Effective when Combined with MDSC Depletion ................................................................................. 242

AIT in Conjunction with Gemcitabine Results in Complete Tumor Rejection and Long-Lasting
Immunological Memory .................................................................................................................. 246

Chapter 2 ........................................................................................................................................ 250

Suppression by MDSC is Contact-Dependent ................................................................. 250

Contact-Dependent Suppression is Independent of CD86, LFA-1, and PD-1L .................................................. 253

Chapter 3 ........................................................................................................................................ 254

MMC-Derived Soluble Factors Drive the Accumulation of MDSC ........................................... 254

GM-CSF is the Main Determinant of MDSC Generation and Survival ........................................... 256

MMC-Derived Soluble Factors or GM-CSF Support the Generation of a Suppressive Ly6G-Ly6C+ MDSC Subset ............. 261

References ........................................................................................................................................ 267
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The structure and binding patterns of the ErbB molecules</td>
</tr>
<tr>
<td>2</td>
<td>Accumulation of granulocytes in the spleen, bone marrow, and blood of FVBN202 mice bearing MMC tumors as compared to tumor free FVBN202 mice</td>
</tr>
<tr>
<td>3</td>
<td>FVBN202 mice bearing MMC tumors have significantly increased numbers of MDSC in both their spleens and bone marrow</td>
</tr>
<tr>
<td>4</td>
<td>The presence of elevated MDSC in tumor bearing FVBN202 mice results in a drastic reduction of lymphocyte precursors in the bone marrow</td>
</tr>
<tr>
<td>5</td>
<td>Accumulation of CD11b+Gr1+ cells in FVBN202 MMC tumor-bearing mice as compared to FVBN202 tumor free mice</td>
</tr>
<tr>
<td>6</td>
<td>Flow cytometry analysis of T cell viability and phenotypes before and after activation with B/I</td>
</tr>
<tr>
<td>7</td>
<td>Anti-tumor efficacy of neu-specific effector T cells before and after activation with B/I and expansion with IL-2</td>
</tr>
<tr>
<td>8</td>
<td>Adoptive immunotherapy using IL-2 expanded T cells is not advantageous unless coupled with the depletion of MDSC</td>
</tr>
<tr>
<td>9</td>
<td>The in vivo depletion of Gr1+ cells alone does not inhibit tumor growth in FVBN202 mice</td>
</tr>
<tr>
<td>10</td>
<td>Levels of MDSC in the peripheral blood of mice receiving anti-Gr1 antibody</td>
</tr>
</tbody>
</table>
Figure 11: Tumor growth is correlated with the persistence of Gr1+ cells in vivo........81

Figure 12: Depletion of Gr1+ cells in vivo restores an anti-neu antibody response in tolerant FVBN202 mice.................................................................83

Figure 13: Lack of antibody response is correlated with the persistence of Gr1+ cells in vivo........................................................................86

Figure 14: Flow cytometry of splenocytes from FVBN202 recipient mice at the termination of the experiment.........................................................88

Figure 15: Flow cytometry analysis of T cells after 7 days of cytokine expansion using either IL-2, IL-7 and IL-15, or a combination of all three, termed “alternating” cytokine expansion.................................................................91

Figure 16: Fold expansion after cytokine treatments...............................................94

Figure 17: CD127 expression on FVB donor lymphocytes.......................................97

Figure 18: The presence of CD4+CD25+FoxP3+ cells in vitro does not inhibit T cell Expansion.................................................................100

Figure 19: Effector function of expanded T cells against MMC target cells..............102

Figure 20: Adoptive immunotherapy using T cells expanded in alternating gamma chain cytokines is only effective when combined with Gr1+ depletions in vivo.................................................................105

Figure 21: The efficacy of Gr1+ depletions is an important factor in the success of adoptive immunotherapy.................................................................108

Figure 22: Only mice receiving Gr1+ depletions exhibit and antibody response against the extracellular domain of HER2/neu............................................110
Figure 23: Flow cytometry of T cell phenotypes from the spleens

Figure 24: Adoptive immunotherapy treatment and with or without Gr1+

dearthions does not increase CD4+ or CD8+ T cells in the spleens of

FVBN202 mice

Figure 25: Adoptive transfer of CD62L+ T cells does not prevent T cell suppression by

MDSC

Figure 26: Gemcitabine inhibits the accumulation of MDSC in the blood of MMC-

challenged FVBN202 mice

Figure 27: Gemcitabine combined with AIT causes complete tumor regression, along

with increased weight and survival of FVBN202 mice

Figure 28: Only mice given AIT and treated with gemcitabine have an antibody response

against subdomain II of the ECD of HER2/neu

Figure 29: FVB mice that have successfully rejected MMC tumors display IgG1 antibody

responses only against subdomain II of HER2/neu

Figure 30: Mice treated with gemcitabine and AIT can reject further challenge with

MMC

Figure 31: Mice receiving AIT and Gemcitabine treatment mount an immune response

against subsequent MMC challenge

Figure 32: FVBN202 mice challenged for a third time with MMC and receiving no

further gemcitabine treatment mount an antibody response against subdomain

II of HER2/neu

Figure 33: Tumor relapse in mouse that received Gemcitabine and AIT and rejected 3
challenges of neu+ MMC cells is of the ANV phenotype…………………..140

Figure 34: MDSC’s suppress CD3/CD28 induced proliferation of T cells from FVBN202 Mice…………………………………………………………………………146

Figure 35: Only MDSC that are Ly6G-Ly6C+ are suppressive against anti-CD3/CD28-induced proliferation………………………………………………………..149

Figure 36: MDSC express low levels of IL-10……………………………………………………………153

Figure 37: Expression of IDO and TGF-β in MDSC subsets……………………………………155

Figure 38: MDSC’s suppress T cell proliferation in a contact-dependent manner………158

Figure 39: CD86 expression on MDSC in MMC tumor-bearing FVBN202 mice is significantly lower than that seen in tumor free FVBN202 mice…………...160

Figure 40: T cells and MDSC highly express LFA-1………………………………………..163

Figure 41: PD-1 is upregulated on CD8+ T cells after re-activation in vitro by MMC cells………………………………………………………………………………165

Figure 42: MDSC’s from FVBN202 mice do not express PD-1 or PD-1L…………….168

Figure 43: MMC-derived soluble factors cause an increase in MDSC in the spleens of FVB mice……………………………………………………………………173

Figure 44: MMC-derived soluble factors cause an increase in MDSC in the blood of FVB mice……………………………………………………………………176

Figure 45: MMC-derived soluble factors cause an increase in MDSC in the bone marrow of FVB mice…………………………………………………………………178

Figure 46: Intradermal injection of MMC-conditioned medium causes a significant reduction in the expression of I-Aq on the surface of CD11b+Gr1+ MDSC
Figure 47: Intradermal injection of MMC-conditioned medium causes a downregulation of the MHC class II related molecule I-Aq on CD11b+Gr1- DC’s in the spleens of FVB mice………………………………………………………………………………181

Figure 48: MMC cells secrete GM-CSF, VEGF, and MCP-1………………………………………185

Figure 49: GM-CSF/IL-6 does not increase MDSC in the spleens of FVB mice………………188

Figure 50: GM-CSF/IL-6 does not increase MDSC in the bone marrow of FVB mice……………………………………………………………………………………………191

Figure 51: GM-CSF/IL-6 does not increase MDSC in the blood of FVB mice…………………193

Figure 52: Treatment with VEGF \textit{in vivo} does not increase levels of MDSC in the spleens of FVB mice…………………………………………………………………………………195

Figure 53: Treatment with VEGF \textit{in vivo} does not increase levels of MDSC in the bone marrow of FVB mice…………………………………………………………………………………198

Figure 54: Treatment with VEGF \textit{in vivo} does not increase levels of MDSC in the blood of FVB mice…………………………………………………………………………………………200

Figure 55: Treatment with MCP-1 \textit{in vivo} does not increase levels of MDSC in the spleens of FVB mice…………………………………………………………………………………………203

Figure 56: Treatment with MCP-1 \textit{in vivo} does not increase levels of MDSC in the bone marrow of FVB mice…………………………………………………………………………………………205

Figure 57: Treatment with MCP-1 \textit{in vivo} does not increase levels of MDSC in the blood of FVB mice…………………………………………………………………………………………207

Figure 58: Culture of whole bone marrow with GM-CSF results in an increase in
CD11b+Gr1- cells.................................................................210

Figure 59: Culture of whole bone marrow with GM-CSF causes an increase in CD86 expression and a decrease in CCR7 expression...............................213

Figure 60: MMC-derived soluble factors and GM-CSF can generate MDSC from CD11b-Gr1- progenitor cells, and can maintain existing MDSC..........................216

Figure 61: MMC-derived soluble factors and GM-CSF can protect newly-derived and existing MDSC from apoptosis.........................................................219

Figure 62: FVBN202 mice contain unique subsets of MDSC..........................223

Figure 63: MMC-derived supernatant and GM-CSF cause the generation of suppressive CD11b+Ly6G-Ly6C+ MDSC from CD11b-Gr1- precursor cells.............227

Figure 64: Culture of CD11b-Gr1- cells with GM-CSF or MMC leads to a decrease in the expression of MHC class I and MHC class II molecules.......................231

Figure 65: GM-CSF and MMC do not cause the proliferation of sorted CD11b+Gr1+ cells in vitro.................................................................233
List of Abbreviations

5-FU.......................... 5-fluorouracil
Ab..............................antibody
Ag..............................antigen
AIT.............................Adoptive immunotherapy
ANV...........................Antigen negative variant
APC...........................Antigen presenting cell
ATP...........................Adenosine triphosphate
B/I..............................Bryostatin and ionomycin
BAD..............................Bcl-xL/Bcl-2-associated death promoter
BM..............................Bone marrow
BrdU..........................5-bromo-2-deoxyuridine
CD..............................Clusters of differentiation
cDNA..........................Complementary DNA
CM..............................Conditioned medium
COX............................Cyclooxygenase
CTL............................Cytotoxic lymphocyte
Cy5..............................Cyanine 5
CYP..............................Cyclophosphamide
DC..............................Dendritic cell
DNA.............................Deoxyribonucleic acid
E:T..........................Effector to Target ratio
ECD.......................Extracellular domain of Neu
ECDII.....................Subdomain II of the extracellular domain of Neu
ECDIV.....................Subdomain IV of the extracellular domain of Neu
EGF.......................Epidermal growth factor
EGFR.....................Epidermal growth factor receptor
ELISA.....................Enzyme-linked immunosorbent assay
FBS.......................Fetal bovine serum
FITC......................Fluorescein isothiocyanate
FoxP3.....................Forkhead box P3
FVB.......................Inbred mouse strain
FVBN202...................Inbred mouse strain overexpressing rat HER2/neu
GAPDH....................Glyceraldehyde-3-phosphate dehydrogenase
Gem.......................Gemcitabine
GHR.......................Growth hormone receptor
GM-CSF..................Granulocyte-macrophage colony stimulating factor
HER2......................Human Epidermal growth factor receptor 2
HLA.......................Human leukocyte antigen
i.d........................Intradermal
i.p..........................Intraperitoneal
i.v..........................Intravenous
ICAM.....................Intracellular adhesion molecule
ICD……………………Intracellular domain
IDO……………………Indoleamine 2,3-dioxygenase
IFN……………………Interferon
Ig……………………Immunoglobulin
IL……………………Interleukin
JAK……………………Janus Kinase
LFA……………………Lymphocyte function associated antigen
Ly6……………………Lymphocyte antigen 6 complex
mAb……………………Monoclonal antibody
MAPK…………………Mitogen-activated protein kinase
MCP-1………………Monocyte chemoattractant protein-1
MDSC…………………Myeloid-derived suppressor cells
MEP…………………..4-mercaptoethylpyridine
MFI…………………..Mean fluorescence intensity
MHC…………………Major histocompatibility complex
MMC…………………Mouse mammary carcinoma
MMTV………………Mouse mammary tumor virus
mRNA…………………Messenger ribonucleic acid
NF-κB…………………Nuclear factor kappa B
NSCLC………………Non-small cell lung carcinoma
O.D…………………..Optical Density
PBS…………………..Phosphate buffered saline
PD-1........................Programmed cell death 1
PD-1L........................Programmed cell death 1 ligand
PE............................Phycoerythrin
PH............................Plekstrin homology
PI............................Propidium iodide
PI3K..........................Phosphotidylinositol 3-kinase
PIP₂..........................Phosphatidylinositol-4,5-bisphosphate
PIP₃..........................Phosphatidylinositol-3,4,5-triphosphate
PTEN..........................Phosphatase and tensin homolog
RB6-8c5......................Hybridoma that produces anti-Gr1 antibody
RNA..........................Ribonucleic acid
RPMI..........................Roswell Park Memorial Institute
RTK..........................Receptor Tyrosine Kinase
RT-PCR........................Reverse transcriptase polymerase chain reaction
SCF..........................Stem cell factor
SD.............................Standard deviation
SEM..........................Standard error of the mean
SH2..........................Src-homology 2 domain
STAT..........................Signal transducer and activator of transcription
TAA..........................Tumor associated antigen
TB............................Tumor bearing
Tcm..........................Central memory T cell
TCR………………..T cell receptor
Tem………………..Effector memory T cell
TF………………….Tumor free
TGF………………..Tumor growth factor
Th1…………………..T helper cell type 1
Th2…………………..T helper cell type 2
TIL………………..Tumor infiltrating lymphocytes
TLR………………..Toll-like receptor
TNF……………….Tumor necrosis factor
VEGF……………..Vascular endothelial growth factor
<table>
<thead>
<tr>
<th><strong>Drug Name</strong></th>
<th><strong>Type of Molecule</strong></th>
<th><strong>Mechanism of Action</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>5-fluorouracil</td>
<td>Uracil analog</td>
<td>Disrupts RNA synthesis; inhibits synthesis of thymidylate</td>
</tr>
<tr>
<td>bevacizumab</td>
<td>antibody</td>
<td>Inhibits angiogenesis by recognizing and binding all isoforms of vascular endothelial growth factor</td>
</tr>
<tr>
<td>carboplatin</td>
<td>DNA alkylating-like agent</td>
<td>Causes DNA cross-linking by binding covalently to the N(^7) position on purines</td>
</tr>
<tr>
<td>celecoxib</td>
<td>Non-steroidal anti-inflammatory drg</td>
<td>Selectively inhibits the cyclooxygenase-2 enzyme</td>
</tr>
<tr>
<td>Cetuximab</td>
<td>Chimeric IgG(_1) monoclonal antibody</td>
<td>Binds domain III of the epidermal growth factor receptor (ErbB1)</td>
</tr>
<tr>
<td>cisplatin</td>
<td>Platinum-based DNA alkylating-like agent</td>
<td>Platinum molecule displaces one of its chloride atoms and covalently binds the N(^7) position on purines, cross-linking two DNA bases.</td>
</tr>
<tr>
<td>cyclophosphamide</td>
<td>Nitrogen mustard alkylating agent</td>
<td>Conversion to its metabolite, aldophosphamide results in DNA cross-linking at the guanine N(^7) position in cells expressing low aldehyde dehydrogenase</td>
</tr>
<tr>
<td>erlotinib</td>
<td>A quinazoline tyrosine kinase inhibitor</td>
<td>Binds to the adenosine triphosphate binding site on the epidermal growth factor receptor, tyrosine phosphorylation and signal transduction</td>
</tr>
<tr>
<td>fludarabine</td>
<td>Purine analog</td>
<td>Prevents DNA synthesis by interfering with the ribonucleotide reductase and DNA polymerase enzymes</td>
</tr>
<tr>
<td>gefitinib</td>
<td>A quinazoline tyrosine kinase inhibitor</td>
<td>Binds to the adenosine triphosphate binding site on the epidermal growth factor receptor, inhibiting tyrosine phosphorylation and signal transduction</td>
</tr>
<tr>
<td>gemcitabine</td>
<td>Deoxycytidine analog</td>
<td>Inhibits the ribonucleoside reductase enzyme when in its diphosphate form and incorporates in DNA thereby inhibiting replication when in its</td>
</tr>
<tr>
<td>Drug</td>
<td>Description</td>
<td>Triphosphate Form</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>indomethacin</td>
<td>Non-steroidal anti-inflammatory drug</td>
<td>Non-selectively inhibits both the cyclooxygenase 1 and 2 enzymes</td>
</tr>
<tr>
<td>irinotecan</td>
<td>A semisynthetic analogue of the naturally occurring cytotoxic quinoline alkaloid, Camptothecin</td>
<td>Inhibits the topoisomerase I enzyme</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>Dual specificity quinazoline tyrosine kinase inhibitor</td>
<td>Binds to the adenosine triphosphate binding sites on both the epidermal growth factor receptor and HER2/neu, inhibiting tyrosine phosphorylation and signal transduction</td>
</tr>
<tr>
<td>Matuzumab</td>
<td>Humanized monoclonal antibody</td>
<td>Binds domain III of the epidermal growth factor receptor and inhibits the domain rearrangement required for exposure of the dimerization arm</td>
</tr>
<tr>
<td>oxaliplatin</td>
<td>Platinum-based DNA alkylation-like agent</td>
<td>Platinum molecule covalently binds the N&lt;sup&gt;7&lt;/sup&gt; position on purines causing DNA crosslinking</td>
</tr>
<tr>
<td>paclitaxel</td>
<td>A taxane: Mitotic inhibitor</td>
<td>Binds and hyper-stabilizes tubulin</td>
</tr>
<tr>
<td>panitumumab</td>
<td>Human monoclonal IgG&lt;sub&gt;2&lt;/sub&gt; antibody</td>
<td>Binds the epidermal growth factor receptor</td>
</tr>
<tr>
<td>pertuzumab</td>
<td>Monoclonal antibody</td>
<td>Binds domain II of HER2/neu to prevent dimerization</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>Humanized monoclonal antibody</td>
<td>Binds domain IV of HER2/neu and inhibits downstream signaling</td>
</tr>
</tbody>
</table>
Abstract

THE ROLE OF MYELOID-DERIVED SUPPRESSOR CELLS IN THE
IMMUNOTHERAPY OF BREAST CARCINOMAS

By Johanna Keeler Morales, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

Major Director: Masoud H. Manjili
Assistant Professor, Department of Microbiology and Immunology

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature
cells at various stages of differentiation. These cells are broadly characterized by the
simultaneous expression of the surface markers CD11b and Gr1 and have been found to
accumulate in large numbers in response to many different tumors in both mice and
humans, including HER2/neu+ breast cancers. The adoptive immunotherapy of cancers
has been a promising field, yet the clinical efficacy of adoptive immunotherapies targeted
against human breast cancers and many other cancers has been extremely limited. Given
the influx of MDSC in tumor-bearing individuals, we hypothesized that these cells were
the reason for the failure of adoptively transferred T cells to effectively reject primary
tumors. Using either monoclonal antibodies or the chemotherapeutic drug, gemcitabine,
we aimed to eliminate MDSC cells \textit{in vivo} to determine if adoptively transferred T cells
would be more effective in the absence of these cells. We further aimed to characterize
the mechanism of T cell suppression by MDSC and the tumor-derived soluble factor(s)
responsible for their accumulation. We have found that the elimination of MDSC \textit{in vivo}
does result in significant tumor inhibition when adoptively transferred T cells are
administered. Furthermore, the use of gemcitabine in conjunction with adoptively
transferred T cells resulted in complete tumor rejection in 100\% of mice and was
accompanied by large antibody titers against HER2/neu as well as strong recall responses
characterized by IFN-\(\gamma\) release and subsequent rejection of further tumor challenges. We
report herein that suppression by MDSC is contact dependent and affects the proliferation
of both CD4+ and CD8+ T cells. The accumulation of MDSC in tumor-bearing mice can
be entirely attributed to tumor-derived soluble factors, with GM-CSF specifically causing
the generation and maintenance of these cells. Our findings suggest that the adoptive
immunotherapy of breast carcinomas in a clinical setting should be combined with the
use of gemcitabine, and that the use of GM-CSF as an adjuvant in cancer vaccines should
be carefully re-evaluated as this cytokine may result in increased MDSC accumulation \textit{in}
vivo.
Introduction

I. The ErbB Family of Receptor Tyrosine Kinases

A. Structure and Function: Breast cancer is the most frequently occurring cancer in women in the United States, with an overall risk of developing breast cancer of 1 in 6, and a risk of developing invasive breast cancer of 1 in 8 (1, 2). Of these cancers, approximately 30% are positive for the overexpression of the HER2/neu oncogene (3), which has been associated with a more aggressive phenotype and poor clinical prognosis (4). HER2/neu, or its 89% homologous rat counterpart, neu (5), is a member of the epidermal growth factor receptor family, or the ErbB family, of tyrosine kinase receptors. The epidermal growth factor receptor (EGFR) was discovered in 1975 as a specific target for epidermal growth factor, previously shown to stimulate the proliferation of epithelial cells (6, 7). In 1978, Stanley Cohen’s group observed that the addition of EGF to epidermoid carcinoma cells increased the incorporation of $^{32}$P in vitro (8). Hence the idea that signals could be transmitted through the phosphorylation of receptors was initiated. In 1984, the peptide sequence of EGFR was discovered to share a high level of similarity with a known avian oncogene, v-erbB (9). Thus, the first connection was made between a known human receptor tyrosine kinase and a sequence exhibiting oncogenic function. In 1985, the discovery of a second member of the EGFR family was published, having been
discovered by Coussens et al using v-erbB as a screening probe (10). This 138,000-dalton polypeptide, encoded by 4.8-kb of mRNA originating from the q21 region of chromosome 17 in humans was found to be widely expressed in many normal tissues including the kidney, liver and colon (10). Furthermore, this paper noted that this newly coined “HER2” gene shared its chromosomal location with that of the previously mapped neu proto-oncogene isolated from a rat neuroblastoma (10). The EGFR family is now comprised of 4 members: ErbB1 (the epidermal growth factor receptor, or EGFR), ErbB2 (or HER2/neu in humans), ErbB3 (HER3), and ErbB4 (HER4) (11). Members of the ErbB family exhibit structural similarity, with an extracellular region composed of four domains, a transmembrane and juxtamembrane region, an intracellular tyrosine kinase domain, and an C-terminal regulatory region (11-13). The extracellular region of the ErbB tyrosine kinases is made up of four sub-domains. Domain I, which is the most distally located from the cell membrane, along with domain III, are homologous members of the leucine rich repeat family, and it is these domains that take part in binding to extracellular ligands (11-15). Domains II and IV, on the other hand, are cysteine-rich domains and do not participate in ligand binding. Rather, domain II acts as a dimerization arm, which, in the presence of ligand, is exposed and contacts the domain II dimerization arm of a second ErbB molecule (Fig. 1A, right) (11-13). In the absence of ligand, however, the ErbB molecule assumes its “tethered” orientation, in which domain II interacts with domain IV in such a way as to block the dimerization interface of domain II (Fig. 1A, center)(11-13). ErbB2, however, is unique in that its domain II dimerization arm is constitutively exposed, thus making it available to heterodimerize with any of the other ErbB family members (Fig. 1A, left) (16).
Unlike the other ErbB receptors, ErbB2 also has no known ligand, which may explain the need for exposure of domain II, since ligand binding to domains I and III in other ErbB molecules results in a conformational change that exposes domain II, resulting in a unique dimerization that is entirely receptor-mediated (14, 15). Since ErbB2 is the only of the ErbB molecules that has no ligand, overexpression of this molecule alone, with no further stimulating factors leads to increased dimerization, both with other ErbB molecules and with ErbB2 itself, and results in increased proliferation and survival (Fig. 1B) (12, 17).

While normal cells express 2-10 copies of the ErbB2 gene, amplification of ErbB2 in breast cancer patients has resulted in as many as 200 copies, correlating with surface expression of up to 2 million ErbB2 molecules, as compared to 20,000-50,000 molecules on the surface of normal breast cells (18-20). Accordingly, experiments have shown ErbB2 to be the preferred dimerization partner for the other 3 ErbB molecules, with tyrosine phosphorylation levels of ErbB molecules 1, 3 and 4 in response to ligand being greatly reduced in the absence of ErbB2 (17). In fact, it has been found that ErbB2 actually enhances tyrosine phosphorylation of ErbB1 in response to its ligand, epidermal growth factor as well as of ErbB3, the only ErbB molecule with no intrinsic kinase activity, and ErbB4 in response to one of their ligands, neu differentiation factor in certain tumor cell lines (21-23). The lack on ligand for ErbB2 can be explained by its crystal structure, which reveals that domains I and III of the extracellular domain are in direct contact, therefore eliminating the need for these two domains to be bridged by a ligand, as they are
Figure 1: The structure and binding patterns of the ErbB molecules

(A) ErbB2 (left) has an exposed dimerization arm (domain II) even in the absence of ligand, whereas ErbB1, 3, and 4 adopt the "tethered" conformation in the absence of ligand (center) which makes the dimerization arm unavailable for binding. Upon ligand binding to ErbB1, 3, or 4, (right) a conformational change occurs that exposes the dimerization arm for binding. (B) ErbB molecules can homodimerize or heterodimerize, with ErbB2 being the preferred dimerization partner for all other ErbB molecules. Figure taken from reference 12.
other ErbB receptors (16) This explains the constitutively active form of ErbB2 independent of a ligand, and justifies the observation that ErbB2 overexpression can lead to cell transformation in the absence of ligand (11, 24).

The FVBN202 mouse model of spontaneously arising mammary carcinomas provides a clinically relevant model for investigating the immunotherapy of neu positive mammary tumors. These mice develop mammary carcinomas within 4-12 months of age as a result of the overexpression of the inactivated form of rat neu oncogene, which lacks the activating mutation that is present in the transmembrane domain of the activated form of the oncogene, in their mammary glands under the regulation of the mouse mammary tumor virus (MMTV) promoter (25). Spontaneously arising mammary carcinomas were then harvested for the development of a neu overexpressing mouse mammary carcinoma cell line, referred to as MMC. The persistent overexpression of the neu oncogene in FVBN202 mice results in mice that are immunologically tolerant to the neu oncoproteins, therefore creating an ideal and clinically relevant model in which to investigate tumors arising from “self” tissues to which immune responses are not robust. In contrast, parental FVB mice do not express the rat neu oncogene and therefore are able to generate a robust immune response against challenge with neu-expressing MMC. These mice subsequently reject MMC, thus generating a pool of T cells with proven effectiveness against these tumors.

**B. Signaling Pathways:** RTK’s have extensive downstream signaling capabilities, and signaling events stemming from ligation of the epidermal growth factor receptor have been extensively studied. Specifically, activation of the ErbB family of RTK’s has been
shown to result in cellular proliferation and pro-survival effects that are mediated through one of three main pathways: the phosphatidylinositol 3-kinase (PI3K) pathway, the Ras-MAPK pathway, or the JAK-STAT pathway. (12, 26). Since ErbB2 does not have a ligand of its own, but has a constitutively active dimerization arm, it frequently functions to augment signaling by the other ErbB molecules, particularly ErbB1 and ErbB3, through heterodimerization. In particular, overexpression of ErbB2 in breast cancers leads to increased activation or constitutively active signaling through the PI3K and the MAPK pathways (27). For example, binding of EGF to ErbB1 and subsequent dimerization results in autophosphorylation of critical tyrosine residues on ErbB1 by its intrinsic kinase function. Subsequently, the Src-homology 2 (SH2) domain of the p85 subunit of PI3K is recruited to the phosphorylated tyrosines ErbB1 (28, 29). Heterodimerization of ErbB2 with ErbB3 also initiates this pathway, as activation of ErbB2 with ErbB3 results in six phosphotyrosine docking sites for the PI3K p85 subunit (27). The p110 subunit of PI3K then generates phosphatidylinositol-3,4,5-triphosphate (PIP₃) from phosphatidylinositol-4,5-bisphosphate (PIP₂). Phosphoinositide-dependent kinase 1 and Phosphoinositide-dependent kinase 2 then phosphorylate and subsequently activate AKT, all of which were recruited to PIP₃ via their plekstrin homology (PH) domains (29). AKT is a serine-threonine kinase that exerts vast effects on cell cycle and survival. Importantly, AKT prevents the translocation of the p21 and p27 cell cycle inhibitors into the nucleus through the phosphorylation of both of these molecules (30, 31). Furthermore, AKT phosphorylates and thereby inhibits the pro-apoptotic BAD molecule as well as activates NFκB, which
activates genes to inhibit apoptosis as well as induce progression through the cell cycle and cellular proliferation (29, 32-36).

A second important means of signaling used by the ErbB family of receptor tyrosine kinases is the mitogen-activated protein kinases, or MAPK, cascade (27). Increased MAPK signaling resulting from ErbB2 overexpression first goes through the small GTPase Ras, which in turn activates Raf, MEK, and ERK, a cascade that has been shown to be indispensable for Ras-mediated oncogenesis (27, 37-39). Hyperactivation of Ras may result from mutation of Ras itself which occurs in 30% of all human cancers (40). Mutated Ras molecules are insensitive to the GTPase-activating proteins which are required to hydrolyze the GTP bound to the active form of Ras to GDP, thus resulting in the restoration of the inactive form of Ras (37, 40). Conversely, hyperactivation of Ras may result from overexpressed or mutationally active ErbB1 or ErbB2 molecules. Of the 30% of breast cancers that overexpress ErbB2, it has been found that 20% of these result from amplification of the ErbB2 gene at the DNA level (41), and mutations in the kinase domain of ErbB1 or ErbB2 can lead to constitutively active signaling (42)(43). Compounding this problem of increased signaling from constitutively active ErbB molecules is the fact that Ras activation and the subsequent MAPK cascade causes results in the transcription of ErbB1 ligands such as TGFα, heparin-binding EGF, and amphiregullin, which then feed back further stimulate ErbB receptor signaling (44, 45). Furthermore, signaling through ErbB1 was reported to be responsible for over half of the total transcriptional effects mediated through the Raf protein (45). Signaling through the MAPK pathway, primarily through the induction of Ras and Raf, is therefore a major
means of signal transduction through the ErbB receptors, with alternations in this pathway linked to a significant number of cancers.

Adding to the diversity of signal transduction through ErbB receptors, Yamauchi et al reported that, although ErbB receptors possess intrinsic kinase ability, that treatment of mice with growth hormone resulted in the phosphorylation of not only the growth hormone receptor (GHR), but also of ErbB1. Further experiments proved that ErbB1 complexes with activated GHR and that ErbB1 was directly phosphorylated by JAK2 which resulted in the association of the adaptor molecule Grb2 to the phosphorylated ErbB1. Importantly, it was shown that the expression of mutant ErbB1 molecules that lacked all intrinsic receptor tyrosine kinase activity had no effect on the phosphorylation levels of ErbB1 in response to growth hormone (46). As the downstream target of JAK molecules is the group of transcription factors designated as signal transducers and activators of transcription (STAT’s), it was also determined that binding of EGF to ErbB1 results in the phosphorylation and translocation of both STAT1 (47) and STAT3 (48). Importantly, STAT3 activation has been shown to maintain the proliferation of cancer cells and is aberrantly activated in 50-90% of human cancers (49-51). Constitutively active STAT3C can cause transformation in normal cells, whereas dominant negative STAT3 can block the oncogenic transformation of cells in vitro (52-54). STAT3 mediates these effects through the activation of genes that promote cellular proliferation such as c-myc and cyclin D1 and suppress apoptosis such as MCl-1 and survivan (55-58). Additionally, vascular endothelial growth factor, which aids in angiogenesis, and matrix metalloprotease-9, which degrades components of the extracellular matrix to allow for invasion, are also activated by STAT3
Furthermore increased levels of phosphorylated JAK2 and STAT3 have been observed ovarian cancer cells and the levels of increased expression were positively correlated with tumor grade and migratory phenotype (61, 62). Additionally, treatment of ovarian cells with EGF was shown to significantly increase IL-6 production and secretion in ovarian cancer cells lines. Since IL-6 is known to signal through STAT3 and is a proinflammatory cytokine associated with chronic disease and promotion of cancer, the upregulation of IL-6 by ErbB1 signaling and resultant increase in STAT3 activity constitutes another mechanism of tumor promotion by ErbB1 (62).

II. Cancer Therapeutics

A. Chemotherapies: Many chemotherapeutic drugs have been developed to target and treat rapidly dividing cancer cells. One of these, 5-fluorouracil (5-FU), is a uracil analog that substitutes a fluorine at the C-5 position of uracil where a hydrogen atom usually resides (63). This treatment was originally devised following the observation that rat hepatomas incorporated the pyrimidine uracil into their RNA at a faster rate than normal tissues. In addition to its disruption of RNA synthesis, 5-FU also inhibits the synthesis of thymidylate, necessary for DNA replication, by blocking the binding site of the thymidylate precursor on the thymidylate synthase enzyme. Despite these effects, 5-FU treatment alone has had a relatively low response rate (10-15%) in patients with advanced colorectal cancer, where it has been found to be most effective (63). Cyclophosphamide, an alkylating agent showing particular potency against lymphocytes, is often used for the treatment of lymphomas and autoimmune diseases (64, 65). Furthermore, the use of
Cyclophosphamide in conjunction with adoptive immunotherapy results in increased
efficacy of adoptively transferred cells because of the elimination of endogenous
lymphocytes, and is able to drive the expansion of a predominantly Th1-skewed response
(66, 67). Cyclophosphamide exists in an inactive form that must be converted to its active
form by oxidase enzymes in the liver. The presence of the enzyme aldehyde
dehydrogenase results in the eventual formation of carboxyphosphamide, whereas low
levels of aldehyde dehydrogenase results in the formation of phosphoramidemustard,
which forms DNA cross-links and leads to cell death (64). Another class of potent, yet
highly toxic anti-cancer drugs are the platinum-based drugs cisplatin, carboplatin, and
oxaliplatin. The platinum molecule at the center of these drugs binds covalently to the N7
position on purines, resulting in DNA cross-linking and cell death (68). These drugs are
often administered in combination with 5-FU. Also administered in combination with
platinum drugs, Gemcitabine has offered several novel mechanisms of anti-cancer action.
Gemcitabine is a deoxycytidine analog that must be converted to its active form by
phosphorylation, which yields successive mono, di, and tri-phosphate forms of the
molecule. The first round of phosphorylation is mediated by deoxycytidine kinase, which
is the rate limiting step of these reactions (69). Once in its diphosphate form, Gemcitabine
can inhibit the ribonucleoside reductase molecule, which is needed for the synthesis of
deoxyribonucleoside diphosphates to be converted to deoxyribonucleoside triphosphates
that will be used in DNA synthesis. Additionally, in its triphosphate form, Gemcitabine is
able to incorporate into DNA and inhibit replication (69). A unique attribute of
Gemcitabine was found in its ability to decrease numbers of myeloid-derived suppressor

cells (MDSC) in the spleens of tumor-bearing mice. These results were confirmed in vitro, with the addition of Gemcitabine to splenocyte cultures causing a significant reduction in MDSC while having no effect on CD4+ or CD8+ T cells or B220+ B cells (70). Importantly, Gemcitabine has been shown to exert effects through the immune system, given that the anti-tumor effects of Gemcitabine seen in immunocompetent mice were completely lost in nude mice in some tumor models. Supporting the role of Gemcitabine on the immune system were the findings that mice given immunogenic tumors resistant to Gemcitabine in vitro still had significantly reduced tumor volumes after Gemcitabine treatment in vivo, as opposed to mice given non-immunogenic, Gemcitabine-resistant tumors, on which Gemcitabine had no effect. Therefore, it is plausible that at least some of the anti-tumor effects of Gemcitabine are mediated through the immune system and that this drug may be an ideal candidate to use in conjunction with immunotherapies such as adoptively transferred T cells (71).

B. Monoclonal Antibodies: The use of monoclonal antibodies for the treatment of ErbB overexpressing tumors is common clinical practice and has shown positive results. Monoclonal antibodies function by disrupting the structure, ligand binding, or dimerization of the ErbB receptors and have the added benefit of recruiting immune effector cells that express Fc receptors. The first monoclonal antibody to be approved by the FDA for use in patients with HER2 overexpressing metastatic breast cancer was Trastuzumab, a humanized antibody directed against domain IV of HER2 which is capable of inhibiting MAPK and PI3K signaling through HER2 (72). Preclinical in vitro studies using trastuzumab indicated its ability to inhibit cell growth, downregulate HER2, reduce VEGF
production, and elicit antibody-dependent cell-mediated cytotoxicity (73). Clinical trials have shown objective responses in 15% and 26% of patients with metastatic breast carcinoma, with clinical benefit being closely correlated with higher expression of HER2 (74, 75). A second monoclonal antibody, pertuzumab, is directed against the domain II dimerization arm of HER2, thus blocking dimerization of HER2 with any of the other ErbB molecules. Although promising in theory, a phase II clinical trial using pertuzumab for the treatment of metastatic breast cancer showed limited benefit, however it must be noted that patients had low expression of HER2 (37). Additionally phase II trials for both hormone refractory prostate cancer and non-small cell lung cancer (NSCLC) have shown no response to pertuzumab (37, 76), although results seem promising against ovarian cancer (37). Cetuximab, which was approved in 2004 for the treatment of refractory metastatic colorectal cancer (77) specifically targets domain III of the EGFR with a higher affinity than its EGF or TNF-α ligands, and therefore blocks their binding (78, 79). A 2004 study using cetuximab monotherapy to treat EGFR positive colorectal cancer resulted in partial responses in 8.8% of patients and 36.8% had stable disease (80). Another phase II study carried out using a combination of cetuximab and the topoisomerase I inhibitor, irinitecan found a significant reduction in the time to disease progression in patients receiving both cetuximab and irinitecan as compared to patients receiving cetuximab alone, although this did not correlate with a significantly increased survival time (78). Since cetuximab is a chimeric IgG1 antibody, anaphylactic reactions in patients prompted the development of humanized and fully human monoclonal antibodies. In 2006, panitumumab, a 100% human antibody, was approved for use in patients with progressive
metastatic colorectal cancer (37). Although this antibody, which also targets the EGFR, is an IgG₂ antibody which does not cause antibody-dependent cell-mediated cytotoxicity, a phase III trial showed an 8% response rate in patients with metastatic colorectal cancer.(81), although phase II trials for panitumumab have not shown promise for patients with NSCLC (82). Matuzumab, currently in phase II clinical trials, is a humanized IgG₁ antibody that binds to domain III of EGFR and instead of blocking ligand directly, sterically inhibits domain rearrangement of EGFR required for receptor dimerization (83). Although matuzumab monotherapy in women with ovarian or primary peritoneal cancers previously treated with chemotherapeutic agents showed not advantage (84), phase I monotherapy trials have shown responses in esophageal squamous cell carcinoma and colorectal cancer. A 23% response rate was seen in a phase I trial combining matuzumab with paclitaxel for the treatment of NSCLC (82). Resistance to monoclonal antibody treatment can arise given the fact that signaling through alternate ErbB receptors can compensate for a loss of signaling in the target molecule. Resistance to trastuzumab has been particularly well documented, showing that the vast majority of patients become resistant to this therapy within one year, with 66%-88% of patients exhibiting resistance to single agent trastuzumab treatment (72, 85). Several factors could influence resistance to trastuzumab and other monoclonal antibody therapies. First, since trastuzumab binds to domain IV of HER2, it is inefficient in blocking the heterodimerization of HER2 in the presence of other ErbB ligands (12, 86). Second, since anti-neu antibody administered in the FVBN202 mouse model has lead to downregulation of neu expression and the emergence of ANV tumors, it is possible that HER2 negative tumor cell clones, selected
under immunological pressure, may also lead to relapse in humans (87). Another prevalent mechanism of trastuzumab resistance is loss of the phosphatase PTEN, which antagonizes the phosphorylation of the PI3K-AKT pathway and has been shown to have increased activity after trastuzumab treatment (72, 85). Patients with HER2 overexpressing breast tumors that are deficient in PTEN show a poor clinical response to trastuzumab. For these patients, treatment with tyrosine kinase inhibitors, which are not dependent upon surface expression of HER2 and can ameliorate signaling directly stemming from ErbB receptors, are an attractive alternative (85).

C. Tyrosine Kinase Inhibitors: The existence of over 58 currently known receptor tyrosine kinases (RTK’s), and the observation that overexpression or mutation of over half of these molecules is associated with hyperproliferative disorders and has been observed in a variety of cancers including gastrointestinal, prostate, ovarian, NSCLC, and breast cancers causes these molecules to be natural targets for cancer therapeutics (12, 26). More specifically, the epidermal growth factor receptor is overexpressed in a substantial percentage of colorectal, pancreatic, lung, and NSCLC and had been found to be mutated in NSCLC as well as in glioblastomas (37). One class of drugs used against the ErbB receptors are tyrosine kinase inhibitors (TKI’s), which can counteract the cascade of events triggered by RTK signaling. One TKI, gefitinib (ZD1839), was approved by the FDA in 2003 for the treatment of NSCLC based on results obtained from phase II trials (26, 88). Although phase I and II trials had indicated that treatment with gefitinib reduced EGFR phosphorylation and exhibited some antitumor activity, later phase II trials failed to achieve statistically significant differences between groups receiving gefitinib and groups
receiving a placebo (88). Therefore, in 2005, the FDA limited use of gefitinib to patients currently receiving the drug or patients enrolling in clinical trials for gefitinib at the time (88). Gefitinib is a quinazoline, a class of inhibitors which works by competing with ATP for its binding site on the EGFR (89). Abolishing the ATP binding site consensus sequence on the EGFR was shown in 1987 to be critical for the kinase function of EGFR as well as leading to eventual down-regulation of this receptor from the cell surface (90). Another quinazoline, erlotinib, was approved by the FDA in 2004 for the treatment of NSCLC and pancreatic cancer, based on increased survival times shown in phase III clinical trials (37). This drug has also shown responses in phase I/II trials for the treatment of metastatic squamous-cell carcinoma of the head and neck and hepatocellular carcinoma when combined with the anti-VEGF antibody, bevacizumab (91, 92), but failed to exhibit advantageous effects as a single agent administered against recurrent glioblastoma, or in combination with bevacizumab against ovarian, peritoneal, or fallopian tube cancers (93, 94). Given the incomplete effectiveness of TKI’s targeting EGFR, TKI’s reactive against several RTK’s are now in clinical trials. One of these, Lapatinib, is a quinazoline with dual specificity for EGFR and HER2 and has reached phase III clinical trials for the treatment of breast cancer, renal cell carcinoma, and head and neck cancer (37). Phase II trials have previously shown lapatinib to be effective in reducing HER2+ metastatic lesions in the brain and have shown clinical benefit against advanced metastatic breast cancers previously treated with Trastuzumab (95, 96). Interestingly, recent in vitro studies have indicated a possible synergy between lapatinib and Trastuzumab, with lapatinib treatment of HER2-overexpressing cells causing an accumulation of inactive HER2 molecules,
thereby rendering these cells more susceptible to the effects of Trastuzumab (97). A major drawback to TKI’s targeted to one or several RTK’s is the development of resistance, which has been observed in the vast majority of patients treated with these drugs (98-100). The development of cells that are resistant to TKI’s could result from any of several factors including the induction of other growth factors such as hepatocyte growth factor (99), through the activation of other RTK’s such as the insulin like growth factor-1 receptor (101) or the fibroblast growth factor receptor (102), through mutations in the RTK that may alter the binding site of the drug (98), or through increased activation and heterodimerization of alternate ErbB molecules, mediated by increased release of ligands such as heregulin and betacellulin which cause heterodimerization of alternate ErbB receptors (103). Of further concern are the in vitro findings that indicate that TKI resistant head and neck squamous cell carcinoma cells displayed a dramatically more aggressive phenotype characterized by increased levels of phosphorylated EGFR, increased proliferation, reduced apoptosis, enhanced angiogenic capacity, and acquired resistance to ionizing radiation (104). Thus, while promising at the onset, the use of TKI’s currently has limited effectiveness combined with a high probability of generating a drug resistant, and possibly more aggressive, phenotype.

**D. Vaccines:** The prospect of creating vaccines for tumors by immunizing patients with either whole tumor cells, peptides from specific tumor-associated antigens, DNA, or dendritic cells loaded with antigen, has been a promising concept, however in most cases has failed to produce substantial clinical results. The exploitation of the immune system to treat cancer has several advantages over the administration of chemotherapies and other
anti-cancer therapeutics, including increased specificity, an accompanying decrease in
toxicity, and the possible development of long term immunological memory to prevent
recurrence. The idea of using the immune system against cancer stemmed from the finding
that human T cells could be reactive to specific antigens expressed or overexpressed on
tumor cells that are not widely expressed otherwise; these are referred to as tumor-
associated antigens (TAA) (105-107). Many TAA’s have been tested experimentally as
vaccines, including the immunodominant HLA-A2-binding epitope of HER2 designated as
E75, which is comprised of amino acids 369-377 (107). Vaccination for the prevention of
breast cancer in the FVBN202 mouse model has been done using heat shock protein 110 as
an immunoadjuvant coupled to the intracellular domain (ICD) of neu. This study showed
the induction of both an IFN-γ response and the secretion of IL-4, indicative of a Th2
response, as well as a strong antibody response, specifically against ICD in immunized
animals. Importantly, vaccination of tumor free FVBN202 animals in the initial phases of
mammary hyperplasia resulted in a significant delay of the onset of spontaneous mammary
tumors (108). In humans, vaccination in an early stage setting has been done is patients
with HER2/neu overexpressing ductal carcinoma in situ (DCIS), a preinvasive form of
malignancy (109). This study used dendritic cells activated with IFN-γ and bacterial
lipopolysaccharide in order to create cells that not only secrete high levels of IL-12, but
may also facilitate the breaking of self-tolerance. Although this study design was
successful in generating HER2/neu reactive, IFN-γ-secreting CD4+ and CD8+ T cells that
were shown to infiltrate breast and caused a reduction in residual DCIS, HER2/neu
expression was also decreased in a majority of patients after vaccination. This indicates
that IFN-γ-mediated immune pressure can lead to the selection of HER2/neu negative variants, which have a less aggressive phenotype than their HER2/neu positive counterparts, but may also cause a relapsed phenotype resistant to HER2/neu targeted therapies (109). Another vaccination strategy in humans which uses the E75 peptide in conjunction with GM-CSF has also shown promise in lowering recurrences rates. Breast cancer recurrence rates were significantly reduced in disease-free patients receiving vaccination as compared to disease-free individuals who were not vaccinated, a difference that was lost after 20 months (110). Mittendorf et al have also used the E75 peptide along with GM-CSF to vaccinate disease-free women with node-positive breast cancer previously treated by standard surgical and chemotherapeutic techniques at high risk of recurrence. Induction of immune responses was confirmed in treated patients and after 22 months, 85.7% of patients receiving vaccination were disease free, as compared to 59.8% disease free patients in the control group, a substantial, albeit not significant, difference (107). Extrapolating this data to include vaccination of disease-free node-negative breast cancer patients yielded an overall 26 month recurrence rate of 8.3% in the vaccinated group versus 14.8% in the control group with mortality rates in the relapsed patients being significantly lower in the vaccinated group as compared to the control group (107). Despite promising results for breast cancer relapse prevention, vaccination of tumor bearing individuals with E75 along with Freund’s Incomplete Adjuvant or with GM-CSF succeeded in inducing CTL responses, but provided no clinical benefit (111, 112). It is thought that the main reason for the failure of vaccination against established tumors is the
low frequency of tumor specific T cells \textit{in vivo} (113), although it seems that vaccination may hold some promise in the area of preventative treatment.

As an alternative to peptide vaccination, the use of matured dendritic cells in vaccines may have substantial clinical benefit given that DC’s in cancer patients are often immature and tolerogenic (114, 115). Dendritic cell based vaccines have therefore been investigated in several ways. Dendritic cells can be pulsed with one or several peptides or with whole cell lysate prior to injection for efficient antigen presentation, they can be transduced with full length cDNA clones or mRNA from known immunogenic peptides, thus resulting in presentation of multiple epitopes of that peptide, or they can be used to generate fusion cells (116). Vaccination with E75 has been evaluated by loading the peptide onto dendritic cells prior to vaccination. While results from these trials showed induction of immune responses and infiltration of CD4+ and CD8+ T cells, there has been an extremely limited or complete absence of any clinical response (117, 118, 118, 119). On the other hand, vaccination in mice using DC transduced with a truncated HER2 caused significant tumor inhibition against challenge with HER2-expressing tumors and caused the development of both cellular and humoral immune responses. Injection of HER2-transduced DC also extended tumor-free periods and reduced the numbers of spontaneous primary tumors and lung metastasis in FVBN202 animals, while DC pulsed with lysate from highly HER2-positive tumor cells had no such effect (120). Various other dendritic cell based vaccines have been developed using multiple antigens, for example, the human telomerase reverse transcriptase, or hTERT, peptide, which is recognized by CTL and expressed on over 85% of all human cancers, has also been used in conjunction with DC’s
A key study in 2000 demonstrated that transfecting DC’s with RNA encoding telomerase and subsequent immunization with these DC’s was able to inhibit the growth of several different tumors in mice. Tumor inhibition was most likely CTL-mediated as in vitro assays showed that T cells from immunized mice lysed TERT+ targets as well as TERT+ tumor cells. Importantly, these results were recapitulated with the use of human cell in vitro (122). Several of these methods have produced promising early results. For example, phase I and II trials loading DC’s with a novel fusion protein consisting of the antigen prostatic acid phosphatase fused to GM-CSF resulted in measurable T cell responses in vitro as well as a significant increase in the median time to disease progression in patients exhibiting immune responses versus exhibiting no response (123). A phase I trial investigating the use of autologous DC’s fused with autologous tumor cells found that these cells were able to stimulate T cell proliferation in vitro and 8 out of 23 total patients vaccinated with fusion cells showed stabilization or regression of disease (124). In addition to dendritic cell based vaccines, DNA vaccines may also have some therapeutic potential. A recent study by Cho et al has shown that immunization of Balb/c mice with a plasmid encoding for one of the dominant epitopes of the rat neu protein along with the TLR 9 agonist CpG generated CD8+ T cell responses in vitro, and caused the regression of established neu+ tumors in vivo. Additionally, vaccination of Balb-neuT mice, which are transgenic for and tolerant to the activated rat neu oncogene, caused substantial delay of onset of spontaneously arising neu+ tumors (125).

E. Adoptive Immunotherapy: Unlike vaccination techniques, the use of adoptively transferred T cells to treat established disease has been an attractive method,
mainly because it allows for very high numbers of effector T cells to be infused into the patient. This method has exhibited some success, particularly in the case of melanoma and in the treatment of patients with cancers expressing viral antigens, such as the Epstein-Barr virus (126). In breast cancer, since one of the predominant sites of metastasis is the bone marrow, it has been shown that the adoptive transfer of reactivated T cells isolated from the bone marrow of patients with pre-existing immune responses resulted in 50% of patients generating tumor-specific IFN-γ response. Patients that did not generate an anti-tumor response were those that were given lower numbers of transferred T cells and were shown to have higher numbers of regulatory T cells in the bone marrow and higher levels of IL-4 and IL-10 in response to tumor antigen (127). These drawbacks highlight the many obstacles of adoptive immunotherapy (AIT) that must be addressed, including the fact that transferred T cells may not properly expand once in vivo, T cells from tumor-bearing individuals are usually of low avidity and occur at a low frequency, and immunosuppressive environments in vivo may inhibit adoptively transferred T cells.

A lack of expansion by transferred T cells in vivo is caused by endogenous T cells acting as “cytokine sinks” (128, 129). Consequently, it has been found that lymphodepletion of the host immune cells results in greater efficacy of the adoptively transferred cells because the newly transferred T cells have greater access to cytokines such as IL-7 and IL-15 that are important for homeostatic proliferation (128, 130, 131). Most AIT regimens therefore use a chemotherapeutic agent such as CYP, which has been shown to create a lymphopenic environment in tumor bearing hosts, prior to transfer (132). Dudley et al have shown that adoptive transfer of expanded autologous tumor infiltrating
lymphocytes (TILs) from melanoma patients pre-treated with CYP and fludarabine for lymphodepletion caused the regression of bulky metastasis at multiple sites in a majority of patients when administered concurrently with the T cell growth factor IL-2 (133). Patients with metastatic breast cancer that was refractory to other treatments received CYP and fludarabine prior to allogeneic hematopoietic stem-cell transplants that had been T cell depleted. Subsequent injections of the isolated T cells 42, 70, and 98 days after stem-cell transplantation resulted in objective tumor regression in 6 out of 16 patients and was associated with donor T cell engraftment (134). Additionally, a single dose of CYP prior to AIT using TILs from ovarian tumors expanded in IL-2 resulted in 1 case of complete regression and 4 cases of regression exceeding 50% of the tumor were reported out of a total of only 7 patients (135). The fact that the latter 2 trials did not use IL-2 injections in vivo indicates that the use of CYP for the creation of lymphopenia may be the most important factor in achieving expansion of transferred T cells. However, homeostatic proliferation within a lymphopenic host may still be ineffective if the expanded T cells are specific for only one antigen. One mechanism of immune escape by tumor cells is antigen loss. Therefore, it is important to adoptively transfer T cells that have specificities for multiple tumor antigens and antigenic epitopes (136). T cells derived from parental FVB mice are therefore ideal candidates for evaluating the efficacy of protocols for expanding the neu-specific T cells in the absence of the nominal antigen, ex vivo since these mice are exposed to whole tumor cells in vivo, generating an immune response effective enough to completely reject MMC cells. Bryostatin-1, a protein kinase C activator, and ionomycin, which increases intracellular calcium levels, are able to cause vast expansion of tumor-
specific T cells, while not expanding naïve, non-sensitized T cells, without the addition of specific tumor antigen by mimicking the signals that result from TCR stimulation (137). T cells expanded in this manner were able to cause regression of MCA-105 pulmonary metastasis in vivo, as well as of established 4T07 and 4T1 mammary tumors (138, 139).

In addition to the activation of donor T cells by bryostatin-1 and ionomycin, the means of T cell expansion by gamma chain cytokines may also have an important effect on T cell generation. The growth factor interleukin-2 has been used to mediate high levels of ex vivo expansion of tumor specific T cells. However, there is evidence that IL-2 expansion may also lead to the induction and maintenance of regulatory T cells, and IL-2 is known to cause activation-induced cell death and the upregulation of Fas/FasL molecules on the surface of T cells (140-143). In particular, the expansion of TILs from breast cancer patients in IL-2 has resulted in predominantly CD4+ cells and low levels of lysis in vitro (113, 144) Alternatively, the cytokine IL-7 is needed for newly arising memory T cells (145). IL-15 has also been known to be important in the generation and maintenance of effector and memory CD8+ T cells. IL-15 can protect effector CD8+ T cells from apoptosis, as well as augmenting the cytotoxic effects of effector CD8+ T cells through the induction of molecules such as IFN-γ, perforin, and granzyme B (146-148). Therefore a better understanding of how these cytokines may work in concert and an optimized expansion protocol are needed to allow for the generation of the most effective pool of anti-tumor T cells to be generated ex vivo.

A need for the generation of high avidity T cells for transfer into tumor-bearing individuals has led to the development of T cells with genetically engineered T cell
receptors (TCRs). The first successful demonstration of this was done by transfecting Jurkat T cells with cDNA clones encoding the α and β chains of the TCR from a line of T cells recognizing the MART-1 TAA of melanoma. The transfected Jurkat T cells were then able to recognize MART-1 targets, demonstrating the feasibility of transferring the specificity of a reactive T cell clone to other T cells (149). This transfer was subsequently accomplished in primary human peripheral blood T lymphocytes, resulting in CD8+ T cell clones that could recognize and lyse melanoma cells in vitro (150). The first clinical trial using TCR gene therapy was completed in 2006. In this study, T cells from melanoma patients that were unresponsive to standard therapies were retrovirally transduced with MART-1 specific TCRs and were adoptively transferred back into patients following lymphodepletion. Out of 17 total patients, 15 showed persistence of transferred T cells for at least 90 days and 2 patients showed complete tumor regression (151). The potential efficacy of this for breast cancer has recently been shown in a mouse model using T cells with chimeric receptors specific for human HER2, which were capable of eliminating pulmonary micrometastases of an aggressive breast cancer line expressing human HER2 (152). However, potential concerns include the mis-pairing of transduced and endogenous TCR α/β chains, resulting in novel combinations that may be autoreactive, and aberrant integration of retroviral vectors potentially leading to malignant transformation (153).

III. Suppressor Cells Abrogate Anti-Tumor Immune Responses

A. Regulatory T cells: In terms of inhibition of adoptively transferred T cells, the potential role of regulatory T cells (Treg) has been investigated. In a murine model of breast cancer, it has been shown that the adoptive transfer of Treg-depleted tumor
infiltrating lymphocytes was more advantageous against established tumors than transfer of total T cell populations and resulted in an increased Th1 response and IFN-γ secretion (154). Immunostimulatory treatments in mice bearing 4T1 mammary tumors were capable of mediating tumor rejection and survival in 65% of treated mice when combined with the attenuation of Treg, an effect mediated by tumor-specific T cell cytotoxicity and increased IFN-γ (155). Furthermore, the removal of CD25+ T cells \textit{in vivo} has resulted in the rejection of B16 melanomas transduced with IL-12, and the transfer of CD25-depleted cells has resulted in complete regression of leukemia in nude mice (156, 157). Clinically, Treg infiltration has been observed in breast cancer patients where they have been linked to more aggressive cancerous phenotypes, poor prognosis, higher histological grade, and the presence of T cells expressing inhibitory molecule B7-H1 (158-160). Increased numbers of CD4+CD25+ T cells were also observed in cases of early-stage NSCLC, late stage ovarian, and metastatic melanomas. These cells have been found to secrete the immunosuppressive molecule TGF-β and suppress the proliferation and cytokine production by tumor infiltrating CD4+ and CD8+ T cells (161, 162). However, several studies contradict the role of Treg in cancer therapeutics. It has been found that administration of IL-2 to lymphopenic patients along with autologous T cell transfer increases the generation of Treg as compared to patients that did not receive IL-2 (143). It was further shown that Treg increase in response to IL-2 treatment in patients with renal cancer or melanoma who were not lymphopenic (163). These findings do not correlate increased Treg levels with clinical outcome and are in direct opposition to previous results showing regression of metastatic melanoma after treatment with CYP followed by AIT and IL-2 (133, 164). Therefore,
although mechanisms have been developed to deplete Treg from patient samples to be used for AIT (165), the role of Treg in AIT currently remains highly ambiguous and this cell type may not cause major inhibition in the case of adoptively transferred T cells. The role of other cell types, such as myeloid-derived suppressor cells, which have been shown to be highly suppressive in many models of mouse and human cancers, should therefore be evaluated in the context of AIT (166-169).

**B. Myeloid-Derived Suppressor Cells:** Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature myeloid-derived cells, which may include macrophages, dendritic cells, and granulocytes, at various stages of differentiation. These cells have thus far been characterized by expression of the surface markers CD11b and Gr1. The accumulation of these cells has been noted in many types of cancers, including the murine colon carcinoma MCA-26, Lewis lung carcinoma, the highly metastatic breast carcinoma 4T-1, the neu+ breast carcinoma MMC and melanoma (B16) (167, 170-173). The critical role of MDSC in cancer patients is underscored by the observation that patients with renal cell carcinoma, melanoma, head and neck cancer, and breast cancer had increased MDSC (174-178). Importantly, increases in the circulating levels of MDSC correlate with clinical cancer stage and metastatic burden of breast cancer (178) Increased numbers of MDSC are associated with enhanced myelopoiesis resulting from factors secreted by the tumor itself, predominantly GM-CSF, which stimulates myelopoiesis, and VEGF, which has been shown to block the maturation of hematopoietic progenitor cells (168). This is supported by the finding that resection of solid fibrosarcoma tumors has resulted in a decrease in the number of CD11b+ Gr1+ cells and in increased number of
CD4+ and CD8+ cells in the spleens of mice (179). In renal cell carcinoma patients, removal of the primary tumor also resulted in the spontaneous regression of pulmonary metastasis (180). In fact, recent data from patients with renal cell carcinoma have demonstrated an increased quantity and intrinsic arginase activity of MDSC. In vitro depletion of MDSC from the PBL of these patients restored the tumor-specific IFN-γ production and T cell proliferation (174). In BALB-neuT female mice, which also develop spontaneous mammary carcinomas, CD11b+Gr1+ cells were observed to increase, not only with age, but also with tumor multiplicity. This increase was shown to be mediated by increased hematopoiesis within the host, as transfer of wild-type bone marrow cells into irradiated BALB-neuT hosts showed increasing numbers of transferred CD11b+Gr1+ cells upon reconstitution as the tumor number of the host increased (181). T cell proliferation in these mice also decreased with the increasing numbers of tumors and MDSC. Expanded MDSC populations have also been shown to have suppressive effects both **in vivo** and **in vitro**, however, the mechanism by which these cells exert their suppression is still controversial.

**i. Mechanisms of T Cell Suppression by MDSC:** Several groups have reported contact-dependent suppression by MDSC. In particular, Gabrilovich et al have shown antigen specific contact-dependency, reporting that while Gr1+ cells do not affect T cell proliferation induced by Concanavalin A, they do have an adverse effect on the number of IFN-γ-producing CD8+ T cells in response to specific peptide in what they determined to be an MHC class I dependent manner (166). It has also been reported that MDSC may work through the down regulation of the TCR zeta chain, an affect that was
only seen in the absence of transwell inserts (182). This affect was not shown to be antigen specific, however, suppression by MDSC was dependent on their derivation from a chronically inflamed environment. This is in agreement with current literature has found that MDSC isolated from tumor bearing mice are suppressive, while MDSC from non-tumor bearing mice have little to no suppressive effect, also supporting the notion that MDSC must be activated, presumably by the IFN-γ secreted by effector T cells, in order to function (167). Further defects in the T cell receptor have been found to be mediated through direct contact between MDSC and T cells resulting in nitration of critical tyrosine residues in the TCR-CD8 complex, causing T cells to be unable to recognize MHC-peptide complexes, an effect caused by the production of reactive oxygen species and peroxynitrite (183).

The function of activated MDSC has also been argued to be dependent on the production of nitric oxide, as well as other soluble factors, by these cells (184). The enzyme nitric oxide synthase (NOS) is responsible for the production of nitric oxide, and there are three main types of this enzyme: inducible NOS, endothelial NOS, and neuronal NOS. Although all three of these have shown some correlation with tumor progression and can be produced by the tumor cells themselves, the inducible form of NOS is of primary importance in MDSC. Nitric oxide can work in a variety of ways, including by the inhibition of JAK1, JAK3, and STAT5 molecules that mediate signaling through the IL-2 receptor (184, 185). Interestingly, inhibition of T cell proliferation by NO has been shown to be potent against T cells stimulated in both antigen-specific and non-specific fashions. Significantly, inhibition of iNOS by L-NMMA has been shown to reverse MDSC mediated
inhibition of naïve T cell proliferation upon stimulation with anti-CD3 and anti-CD28, as well as in T cells stimulated with specific peptide or Concanavalin A (170, 184). In all of these cases, IFN-γ was required for the production of NO by MDSC.

In addition to nitric oxide, indoleamine 2,3-dioxygenase (IDO) has also been implicated as one of the effector molecules of MDSC. IDO is an immunomodulatory enzyme that can also be secreted by alternatively activated macrophages as well as dendritic cells and by a variety of malignant human cancer cells. IDO is the rate limiting enzyme of the kynurenine pathway of tryptophan catabolism, metabolites from which cause T cell apoptosis. Thus, an excess of IDO facilitates the breakdown and subsequent depletion of the essential amino acid tryptophan, resulting in inhibition of T cell activation and proliferation. IDO is therefore important in maintaining peripheral tolerance and has also been implicated to play a significant role in a mother’s tolerance to her fetus and in the suppression of transplant rejection (186). Importantly, much like NO, IDO production in APC’s is initiated by IFN-γ, and therefore would be a logical mediator of suppression by IFN-γ activated MDSC. In a study using the neu transgenic mouse model, it was shown that the IDO inhibitor 1-methyl–tryptophan (1-MT), when combined with a chemotherapeutic agent such as cyclophosphamide, resulted in a reduced tumor volume, an effect that was shown to be dependent on endogenous T and B cells. The lymphocyte-dependent effect of 1-MT was not, however, a result of direct effects on T and B cells, as proliferation of these cells was unaffected in vitro; thus it was the inhibition of IDO+ DC by 1-MT that resulted in the restoration of T cell proliferation (187).
A third soluble mechanism of MDSC suppression is the expression of arginase I, which depletes L-arginine from the environment and thus causes T cell dysfunction by inhibiting expression of the CD3 zeta chain (188). This finding correlates with reports of reduced TCR zeta chain expression in clinical studies done on patients with breast cancer, melanoma, and gastric cancer (182, 189-191) and the fact that increased levels of arginase activity have been found in CD11b+ cells in the peripheral blood of renal cell carcinoma patients and was concurrent with a decrease in serum arginine and increased serum ornithine, a metabolic breakdown product of arginine (174). It was later shown that the production of arginase by MDSC was a result of tumor-derived soluble factors from the injected Lewis Lung Carcinomas (3LL). In particular, prostaglandin E2, produced through the action of COX-2, which is constitutively expressed along with COX-1 in 3LL tumors, was found to induce arginase 1 expression in MDSC via the E-prostanoid 4 receptor. Specific inhibition of COX-2, but not COX-1, completely ablated the induction of arginase 1 in MDSC in this model (173).

**ii. MDSC and COX-2:** COX-2 has proven to be a key molecule in promoting inflammation and tumor induced angiogenesis, and is expressed by inflammatory cells after exposure to proinflammatory cytokines. COX-2 overexpression can also be found in many human cancers including breast carcinoma and is believed to be upregulated in tissues in response to carcinogens such as ultraviolet light, nicotine, and *Helicobacter pylori*. Expression of this enzyme leads to the production of prostaglandin-E2 from arachidonic acid, which subsequently upregulates the Th2 response (resulting in increased pro-angiogenic factors such as IL-6 and VEGF) while simultaneously
downregulating the Th1 response, leading to decreased apoptosis in tumor cells through the induction of bcl-2 (192). Indeed, COX-2 expression and activity has been correlated with increased aggressiveness in breast cancer. It has been shown that COX-2 protein expression and activity were greatly increased in a metastatic breast cancer cell line as compared to a nonmetastatic cell line (193). This difference was shown to be mediated by increased methylation of the COX-2 promoter region in DNA from the nonmetastatic carcinoma. Conversely, the promoter region in metastatic breast carcinoma lines remained unmethylated. Treatment of cells with the DNA demethylating agent 5-aza-2’-deoxycytidine correlated with an increase in COX-2 expression and activity (193).

In accordance with the correlation between increased COX-2 expression and the development of cancer, it has been observed that the use of nonsteroidal anti-inflammatory drugs (NSAIDs) is linked with a decreased mortality rate from human breast and colon cancers (194, 195). Likewise, several studies have indicated that use of COX-2 inhibitors slow the progression of tumors. Rozic et al showed that both the non-selective inhibitor indomethacin and the selective inhibitor NS-398, resulted in an inhibition of the migration, invasion and angiogenesis of a highly metastatic C3L5 mammary tumor, while the selective COX-1 inhibitor valeryl salicylate did not have a significant effect (196). Kundu and Fulton confirmed the effectiveness of indomehtacin, and another COX-2 inhibitor, celecoxib, against tumor volume, incidence, and metastasis in the murine mammary tumor cell line 410 but also found the COX-1 selective inhibitor, SC560, to be effective (197). Here, the discrepancy of the effectiveness of COX-1 inhibition lies in the fact that the cell line used in the first study expressed very low levels of COX-1 whereas the 410 mammary
carcinoma of the latter study expressed higher levels of this enzyme and therefore would be more susceptible to its inhibition.

Also of significance is the finding that administration of celecoxib greatly reduced the number of 1,2-dimethylhydrazine induced intestinal tumors and that this decrease in tumor burden was correlated with a substantially decreased number of CD11b+ Gr1+ splenocytes in the mice that were given celecoxib in addition to 1,2-dimethylhydrazine versus mice that were given 1,2-dimethylhydrazine alone. Additionally, this study showed greatly decreased mRNA levels of NOS-2, arginase, and COX-2 in the spleens of animals given celecoxib treatment versus animals given 1,2-dimethylhydrazine alone (198). These findings and others, which have indicated that COX-2 expression in tumors is also linked to increased numbers of CD11b+ Gr1+ MDSC and these cells can work through a variety of mechanisms, including through the production of arginase 1, which is a direct result of prostaglandin E2 production via COX-2 expressing tumors, provides some interesting insights into the crosstalk and feedback mechanisms that exist between tumor-derived soluble factors and myeloid-derived suppressor cells (173). Interactions between known tumor-derived soluble factors, as well as yet-unknown factors, should be thoroughly investigated in order to determine the multi-factorial parameters of MDSC activation and function within the tumor-bearing host.

### iii. Mechanism of MDSC Induction:
In addition to its role in tumorogenesis through activation by ErbB1, STAT3 is also believed to downregulate the expression of proinflammatory cytokines and can have a profound impact on the immune response to
cancer (199). It has been found that blocking STAT3 causes an increase in several proinflammatory cytokines in melanoma, colon carcinoma, and breast carcinoma cell lines (199). These proinflammatory cytokines proved essential in causing the activation of potent dendritic cells in the context of the innate immune response, and transduction of tumor cells with a dominant negative form of STAT3, STAT3β, caused increased infiltration of immune cells to the tumor site in addition to causing the apoptosis of B16 melanoma cells. Increased apoptosis induced by blockade of STAT3 is caused by STAT3 expression upregulating the anti-apoptotic proteins BCL-XL, MCL1 (myeloid cell leukemia-1, a member of the Bcl-2 family that controls mitochondrial integrity), myc, and survivin. Tumor expression of STAT3 was additionally found to inhibit the maturation of dendritic cells, particularly because of the fact that STAT3 expression upregulates immunosuppressive molecules such as IL-10 and TGF-β. Binding of IL-10 to its receptor on dendritic cells can subsequently activate STAT3 within the dendritic cell, and cause the downregulation of critical maturation factors such as MHC Class II molecules, and the co-stimulatory molecules CD80 and CD86 (199, 200). Thus, the activation of STAT3 in tumor cells leads to and maintains the activation of STAT3 in MDSC. This was also shown by Nefedova et al who demonstrated that it was Jak2 activation by tumor-derived soluble factors, particularly M-CSF and to a lesser extent VEGF and IL-10 secreted by C3 and CT26 cell lines, that lead to the hyperactivation of STAT3 in MDSC (201). IL-6, which has anti-apoptotic and pro-angiogenic effects and can regulate the differentiation of myeloid lineages and dendritic cells, has also been shown to activate STAT3 (202). In fact, COX-2 overexpression in a human non-small cell lung carcinoma cell line caused
increased STAT3 phosphorylation of both the tyrosine 705 and the serine 727 residues, which was at least partly mediated by increased IL-6 expression, in these cells (203). Phosphorylation of the tyrosine 705 residue of STAT3 results in its dimerization and nuclear translocation, whereas phosphorylation of serine 727 is believed to cause the subsequent activation of transcription (199). The effect of tumor-derived soluble factors on STAT3 was confirmed in vivo by the fact that STAT3 DNA binding was significantly increased in Gr1+ cells from CT-26 tumor bearing mice over that of control mice. One mechanism by which STAT3 activation causes increased MDSC is by maintaining a high rate of proliferation amongst these cells, however, further studies should be done to investigate other potential surface molecules or soluble factors upregulated in these cells as a result of STAT3 hyperactivation that may cause a positive feedback loop which initiates the accumulation of more MDSC either by contact-dependent mechanisms or by actively blocking the maturation of newly recruited hematopoietic cells, in addition to the increased proliferation of existing MDSC (201).

MDSC can act in a variety of ways to cause suppression of anti-tumor immune responses including, but not limited to, the secretion of soluble factors such as nitric oxide and arginase I. There are also a variety of mechanisms by which the actions of MDSC may be induced, regulated, or altered. Adding to the complexity are the means by which MDSC may be induced and regulated. Several groups support the idea of activation of MDSC by IFN-γ, which requires STAT1 to exert its effects. Meanwhile, recent data also supports an important role for STAT3 in MDSC function. The data are therefore seemingly contradictory given that the effects of STAT1 and STAT3 have been shown to contradict
one another in terms of cellular proliferation as well as apoptosis (204-206). Suppressive activity by these cells may therefore require a balancing act between opposing signals, or alternatively, may require that one pathway be able to overcome another in order to maintain a suppressive phenotype. The role that individual cytokines and chemokines play in inducing and maintaining the suppressive phenotype of MDSC therefore is of critical importance and warrants further investigation in varying tumor models, particularly as the tumor microenvironment and subsequent cytokine profiles will vary in different tumor models.

iv. MDSC and Soluble Factors: A correlation between tumor burden and increased MDSC suggests that tumor-derived factors may cause accumulation of MDSC in cancer patients. Pan et al found that abrogating the secretion of stem-cell factor, or blocking its receptor, ckit, reduced MDSC expansion in mice bearing MCS26 colon carcinomas (207). In a transgenic model of breast carcinoma which expresses the activated form of the neu oncogene, VEGF serum levels were found to correlate with tumor multiplicity and progression, as well as accumulation of MDSC (181). Continuous VEGF administration in mice has been shown to result in the inhibition of dendritic cell development as well as an increase in Gr1+ cells and blocking VEGF in vitro prevents the negative effects of tumor-derived soluble factors on dendritic cells (208). In humans, high levels of VEGF in the plasma of patients with head and neck cancer, lung cancer, and breast cancer were closely correlated with a decrease in functional dendritic cells in the peripheral blood concurrent with an increase in circulating immature myeloid cells (209). GM-CSF has also been linked to the accumulation of MDSC, both in murine models through GM-CSF transduced tumor cells lines (167), and in humans, where a correlation was made between levels of circulating
CD34+ myeloid cells in patients with head and neck carcinoma and the ability of the tumor cells to secrete GM-CSF (210). Interestingly, a review compiling the results from multiple clinical trials using GM-CSF has found that the dosage of GM-CSF was paramount, with lower doses (40-80µg) eliciting an immune response, whereas higher doses showed no advantage (211). For example, melanoma patients receiving peptide vaccination along with either 100 or 500µg doses of GM-CSF for 6 days showed a decrease in the induction of specific T cell responses (212), which was associated with increased levels of MDSC (213). In a mouse model of melanoma, increasing the concentration of GM-CSF beyond the therapeutic dose of 300ng/10^6 cells/24 hours resulted in decreased survival and increased CD11b+Gr1+ cells in multiple organs (214). However, there is no direct evidence identifying the major tumor-derived soluble factors that could induce generation of MDSC from bone marrow progenitor cells and/or support MDSC survival.

v. Subsets of MDSC and the Ly6G Molecule: Given the broad range of heterogeneous cells commonly defined as CD11b+Gr1+ MDSC, recent studies have explored more specific and extensive phenotyping for these cells. These studies identify different epitopes of the Gr1 molecule, namely Ly6G and Ly6C in distinguishing subsets of MDSC that may be more prone to expansion and/or suppression in various tumor models (171, 215, 216). The Ly6 family members are cysteine-rich molecules that are usually glycosyl phosphatidylinositol (GPI)-anchored to the cell surface and it has recently been reported that expression of Ly6G correlates with a granulocytic phenotype, while expression of Ly6C correlates with a monocytic phenotype (171, 215). It is important to note, however that the subsets have not been identified in FVBN202 mice bearing neu+ (MMC) tumors. Given the discrepancies between subset phenotypes, expansion, and
suppressive capabilities in various models, we believe it is important to investigate the specific subsets of MDSC present in FVBN202 MMC tumor bearing animals. Furthermore, given differences in the profiles of soluble factors secreted by various tumors, we thought it imperative to identify the specific soluble factor(s) involved in the generation and accumulation of MDSC, MDSC subsets, and the function of these subsets in mice with neu+ tumors.

Although MDSC in mice are becoming fairly well-defined, MDSC phenotypes in humans are still a topic of debate. MDSC were first broadly defined as being negative for lineage markers indicative of mature lymphocytes or myeloid cells including CD3, CD19, CD57 and CD14 (178, 209). These cells also consistently express the myeloid marker CD33, and in tumor-bearing individuals this is often the predominant marker used for MDSC isolation (178, 217, 218). These cells are also frequently identified as being HLA-DR negative or low (178, 218, 219). However, the presence of other markers has varied significantly between different cancers, with MDSC in renal cell carcinoma patients being described as CD15+ CD14- while identification of MDSC in patients with hepatocellular carcinoma did not use CD15 as a marker, but found MDSC to be CD14+ and HLA-DR negative or low (218, 219). MDSC in patients with breast cancer have been defined as being lineage negative or low, HLA-DR- CD33+ and CD11b+ (178) The phenotypic markers used to identify MDSC not only vary between different types of cancer, but also between groups, as Rodriguez have recently identified a subtype of arginase I producing MDSC in patients with renal cell carcinoma as expressing high levels of CD66b, CD11b, and VEGFR1 while expressing low levels of CD62L and CD16 while Ko et al have
recently identified MDSC in renal cell carcinoma patients with the more conventional CD33+ HLA-DR- CD15+ CD14- designation (218, 220). Since MDSC can use a variety of mechanisms of suppress T cell activation and proliferation, and since MDSC, being derived from multi-potent bone marrow cells and existing at various stages of differentiation, are inherently heterogeneous, the identification of specific markers of MDSC that correlate with specific function would offer the most advantageous means of developing treatments to counteract the detrimental effects of these cells.
Materials and Methods

Mouse Model:
Parental FVB (Jackson Laboratories) and FVBN202-transgenic female mice (Charles
Riveer Laboratories) were used between 6-10 weeks of age throughout these studies.
FVBN202 mice overexpress an unactivated rat neu transgene under the regulation of the
MMTV promoter (25). These mice develop pre-malignant mammary hyperplasia similar to
ductal carcinoma in situ (DCIS) prior to the development of spontaneous carcinoma (221).
These studies have been reviewed and approved by the Institutional Animal Care and Use
Committee (IACUC) at Virginia Commonwealth University.

Tumor cell lines

The MMC cell line was established from a spontaneous tumor harvested from an
FVBN202 transgenic mouse as previously described (136). The antigen negative variant
(ANV) cell line was derived from a relapsed MMC tumor in the FVB strain as previously
described and is characterized by a loss of neu expression (136). Both cell lines were
maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Mice were
challenged with 3-5x10⁶ MMC cells intradermally (i.d.) where indicated.

Flow cytometry
Flow cytometry analysis was performed as previously described by our group (136). Briefly, splenocytes or bone marrow cells were homogenized into a single cell suspension and red blood cells were lysed using 1X ACK Lysis buffer for 5 minutes. Cells were washed and resuspended in 1XPBS with 2% FBS and 0.0065% sodium azide. Cells were counted and $10^6$ cells were aliquoted into each sample tube. Non-specific binding to Fc receptors was blocked with anti-CD16/CD32 antibody (Biolegend) for 20 minutes on ice. The following primary antibodies were purchased from Biolegend and used to stain the surface antigen CD11b (FITC or PE, clone M1/70), Gr1 (PE or PE/Cy5, clone RB6-8C5), CD4 (PE or PE/Cy5, clone GK1.5), CD8 (PE or PE/Cy5, clone 53-6.7), CD44 (PE, clone IM7), CD62L (FITC, clone MEL-4), CD69 (FITC, clone H1.2F3), CD25 (FITC, clone PC61), LFA-1 (FITC, clone 2D7), CD127 (FITC, clone SB/199), CD86 (PE, clone YTS156.7.7), PD-L1 (PE, clone 105.9G2), I-A$^b$ (Biotinylated, clone KH116), and CCR7 (PE/Cy5 clone 4B12). Primary antibodies purchased from BD Pharmingen were against the surface antigens CD8a (FITC, clone 53-6.7), PD1 (PE, clone J43), H-2D$^b$/H-2L$^q$ (Biotinylated, clone KH117), and Ly6G (FITC, clone IA8). A purified mouse monoclonal antibody against rat neu was purchased from Calbiochem (anti-c-ErbB2/c-Neu). All primary antibodies were added at the manufacturer’s recommended concentration and incubated on ice in the dark for 20 minutes. Cells were washed twice and secondary antibody added where applicable (FITC anti-mouse IgG and PE/Cy5 Streptavidin, both from e-bioscience) and incubated and washed as before. Cells were fixed with 1% paraformaldehyde or were washed again in 1X Annexin V buffer (BD Pharmingen) and the Annexin V staining protocol was followed for the addition of Annexin (FITC or PE)
and/or propidium iodide (BD Pharmingen). Whole blood was obtained by retro-orbital bleed. 50X10⁶ whole blood cells were used per sample and were blocked and stained as described above, and subsequent red blood cell lysis with 1X ACK. Samples were run on a Beckman Coulter FC 500 and analyzed using Expo 32 or Summit version 4.3 software.

**Cytotoxicity assay**

Neu-specific effector lymphocytes were cultured with MMC at 10:1 and 20:1 E:T ratios in complete medium (RPMI 1640 supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS) and 20 U/mL recombinant IL-2 (Preprotech) in 6 well culture dishes. After 24 hours, 3 mL fresh media was added to the existing 3 mL of media. After 48 hours, cells were harvested and stained for neu (anti-c-ErbB2/c-Neu, Calbiochem), Annexin V and PI according to the manufacturer’s protocol (BD Pharmingen). Flow cytometry was used to analyze the viability of neu positive cells.

**IFN-γ ELISA**

Effector lymphocytes were cultured in complete medium at a 10:1 ratio with irradiated MMC cells or ANV cells (15,000 rad) or in medium alone for 24 hours. Supernatants were collected and stored at -80°C until used. IFN-γ was detected using a Mouse IFN-γ ELISA Set (BD Pharmingen) according to the manufacturer’s protocol.

**Expansion of effector T cells from FVB mice**

FVB parental mice were inoculated with 5X10⁶ MMC cells and splenocytes harvested after 20-30 days. Splenocytes (10⁶ cells/mL) were stimulated in complete medium
containing 15% FBS with bryostatin-1 (5 nM) and ionomycin (1 µM) along with 80 U/mL of IL-2 for 16 hours. Cells were washed 3 times and cultured at 10^6 cells/mL in complete medium, Cells expanded in IL-2 alone received 40 U/mL of IL-2, while cells expanded in IL-7 and IL-15 received 10ng/mL of each cytokine. Media was changed and fresh cytokines added every other day for a total of 7 days. Cells expanded with alternating gamma chain cytokines were cultured on day 1 with 10 ng/mL IL-7 and 10 ng/mL IL-15. On day 2, 40 U/mL of IL-2 was added. Medium was changed on days 3 and 5, each time culturing with 10 ng/mL each of IL-7 and IL-15 and cells were harvested for infusion on day 7. All cytokines were from Peprotech. Aliquots of cells were taken at indicated time points and samples were stained with combinations of the following antibodies and assessed by flow cytometry: FITC-CD25, FITC-CD62L, FITC-CD8, PE-CD44, PE-CD69, PE-CD8, PE/Cy5-CD4, PE/Cy5-CD8, PE/Cy5-CD69 from Biolegend and PE-CD25, PE-CD4 from BD Pharmingen. All antibodies were used at the manufacturer’s recommended concentration.

Adoptive immunotherapy

For MDSC depletion and CD62L+- AIT studies: twenty-four hours prior to AIT, FVBN202 mice were treated with Cyclophosphamide (CYP, 100 mg/kg) by intraperitoneal (i.p.) injection in order to induce lymphopenia. Mice were challenged i.d. with 3-5X10^6 MMC cells and then received 70X10^6 T cells by tail vein injection later the same day. For AIT with Gemcitabine studies: CYP (100 mg/kg given i.p.) was given 48 hours prior to adoptive transfer and 3X10^6 MMC cells were administered i.d. 24 hours prior to adoptive
transfer. AIT was then administered on Day 0 as before. In all cases, tumor growth was monitored by digital caliper and tumor volumes were calculated by: 

\[ V(\text{volume}) = \frac{L(\text{length}) \times W(\text{width})^2}{2}. \]

Blood was collected from the orbital sinus periodically to determine antibody responses and levels of CD11b+Gr1+ cells by flow cytometry. At the termination of the experiment, splenocytes were harvested and stained for activated T cells or used in IFN-\(\gamma\) release assays, as indicated.

Depletion of MDSC in vivo

For AIT using IL-2 expanded T cells, LEAF purified monoclonal antibody against the surface antigen Gr1 (clone RB6-8C5) was ordered from Biolegend and 300\(\mu\)g was administered i.p. starting on day 6 after tumor challenge and continuing every 3 days for the indicated time period. For AIT using alternating gamma chain cytokine expanded T cells, anti-Gr1 antibody was purified from the RB6-8C5 hybridoma by collecting supernatant from the CELLine CL 1000 flask (IBS Integra Biosciences) according to the user manual. Supernatant was stored at -80\(^\circ\)C until IgG purification using a MEP-Hypercell column. Where indicated, mice were injected i.p. with 250\(\mu\)g of purified anti-Gr1 antibody for a total of 6 times at 3-day intervals, followed by a final injection of 200\(\mu\)g on day 25. In all cases, depletion was verified by flow cytometry of the peripheral blood for the CD11b and Gr1 surface markers. The dose of anti-Gr1 antibody was proven completely effective in mice bearing MMC tumors that were 25mm\(^3\) or smaller.
**Gemcitabine Treatment**

Mice receiving CYP and MMC challenge were given 75mg/kg of Gemcitabine i.p. on days 6, 11, and 16 after MMC challenge. Gemcitabine injections after re-challenge with MMC were also 75mg/kg each and administered on the days indicated. Flow cytometry of peripheral blood showed complete inhibition of MDSC after 2 injections.

**Recombinant neu protein**

The cDNAs coding for sub-domain II and sub-domain IV of the extracellular domain of rat neu (ECDII: 187-332 aa and ECDIV: 503-642 aa) were amplified by PCR using the following primers: ECDII: 5’- GTGGAAATTCACCAATCGTTCCGGGC-3’ (sense) and 5’-CTCCTCGAGACAACGCTGTTGCCGC-3’ (antisense); ECDIV: 5’-ACGAAGCTTGAGGACTTGTGCGTCTCG-3’ (sense) and 5’-AGTGGATCCTCGTTCATCCAGATCCAC-3’ (antisense). Restriction sites are underlined. The resulting ECDII fragments were cleaved with EcoRI and XhoI restriction enzymes and ligated into the EcoRI-XhoI fragment of pRSET B, while fragments from ECDIV were cleaved with HindIII and BamHI restriction enzymes and ligated into the HindIII-BamHI fragments of pRSET B to generate the constructs. The cDNA for full length ECD (23-654 aa) was amplified using the primers 5’-ATTGGTACCAACCATCGTGTACCAGCACA-3’ (sense) and 5’-TGCAGAATTCGTCACCAGGGGCTGGCCTCT-3’ (antisense) with underlined restriction sites cleaved with KpnI and EcoRI. Resulting fragments were ligated into the KpnI-EcoRI fragment of pRSET B. The recombinant proteins were expressed in *E.coli* using IPTG as
an inductor of expression. Purification of His-tagged protein was performed under
denaturing conditions using the Guanidinium Lysis Buffer. After elution, proteins were
dialyzed in 20 mM Tris, pH 9.0 overnight (4°C). Dialyzed proteins were concentrated
using 10,000 MW cut-off columns (Viva Spin), filter-sterilized and protein concentration
was determined using Bradford assay.

**ELISA**

Blood was collected from mice via the retro-orbital sinus, allowed to sit at room
temperature for 10 minutes, and then spun for 10 minutes at 10,000 rpm. Serum was
harvested and stored at -80°C until used. For measuring the antibody response against neu,
96 well plates were coated with 10 µg/mL of full length ECD or 4 µg/mL of subdomain II
(ECDII) or subdomain IV (ECDIV) of ECD and incubated overnight at 4°C. Plates were
washed with PBS+0.05% Tween-20 and blocked with 2% skim milk for one hour. After
washing, 5-fold serial dilutions of the sera were added (100 µl/well) and incubated for 2
hours at room temperature. Horse-radish-peroxidase (HRP)-conjugated anti-mouse IgG1
from Caltag was added at a 1:2000 dilution for 1 hour. Plates were washed and reactions
developed by adding 100 µl/well of the TMB Microwell peroxidase substrate (Kierkegaard
& Perry). The reaction was stopped with 2 M H₂SO₄, and the O.D. read at 450 nm. Mean
antibody titers were then calculated where indicated.

**Isolation of MDSC in vitro**

Gr1+ cells were isolated using an EasySep PE Selection kit from StemCell Technologies.
The protocol from the manufacturer was followed using splenocytes homogenized from
MMC tumor-bearing FVBN202 mice labeled with 2 µg/mL of PE-Gr1 or PE-CD11b antibody from Biolegend. Purity of Gr1+ cells was confirmed by flow cytometry and was >90%.

In vitro proliferation assays and BrdU labeling

Splenocytes or sorted CD11b+Gr1+ cells were cultured in 96 well plates. For T cell stimulation assays using splenocytes, plates were coated with 10 µg/mL of anti-CD3 (BD Pharmingen) and were washed 3 times with PBS after 24 hours to remove any unbound antibody. Cells were brought to a concentration of 1X10^6 cells/mL in complete media and 2X10^5 cells were added to each well. Cells were labeled by adding 10 µM BrdU (5-bromo-2-deoxyuridine, BD Pharmingen) directly to the culture medium. Soluble anti-CD28 antibody (BD Pharmingen) was also added to wells for T cell stimulations at 1µg/mL. For sorted CD11b+Gr1+ proliferation assays, 100ng/mL of GM-CSF (Peprotech) was added. Cells were allowed to proliferate for 72 hours at 37°C, 5%CO₂. Staining for BrdU was done following the protocol from the manufacturer (BD Pharmingen) using the FITC-conjugated anti-BrdU flow kit. Where indicated, MDSC were depleted from the splenocyte populations using the PE Selection protocol above with either PE-Gr1 or PE-CD11b antibodies (Biolegend). Isolated MDSC were added to wells where indicated at a 1:2 MDSC to splenocyte ratio either in the absence of a transwell insert, or in the top chamber of a transwell insert with 8.0um pore (Corning Life Sciences). MDSC sorted on the basis of Ly6G and Ly6C expression were added to splenocytes at a 1:2 MDSC to splenocytes ratio where indicated and labeled with BrdU and stained as above.
**MMC-derived supernatants**

MMC-derived conditioned medium was generated by culturing 10X10^6 MMC cells in 10mL of media for 24 hours in RPMI1640 supplemented with 10% FBS. Supernatant was then removed and concentrated to a volume of 600µL using 10,000kDa molecular weight cut-off columns (Viva Spin). Concentrated supernatant or control medium was then injected i.d. into FVB mice (n=3, 200µL per mouse). Injections were repeated once per day on 3 consecutive days; mice were then sacrificed on day 4 and spleens, blood, and bone marrow were subjected to flow cytometry analysis of CD11b and Gr1 levels, as described above.

**Multiplex cytokine array of MMC supernatants**

MMC cells were cultured at a concentration of 10^6 cells/mL for 24 hours. Supernatants were harvested and sent to Allied Biotech, Inc. for Multiplex array analysis in a blinded fashion.

**Cell sorting**

Bone marrow cells were harvested from naïve FVBN202 mice and stained for surface expression of CD11b and Gr1 as described. Cells were kept in sterile RPMI1640 with 10% FBS throughout. Cells were sorted on a Cytomation MoFlo by gating on the granulocyte region and then sorting CD11b+Gr1+ and CD11b-Gr1- cells. MDSC subsets were triple sorted from the spleens of tumor-bearing mice by first staining with anti-Ly6G, followed by anti-CD11b and anti-Gr1. Cells were gated on CD11b+ cells from within the gated granulocyte region, and subsequently sorting this population based on expression of Ly6G.
and Ly6C. After sorting, an aliquot of each population was restained for the CD11b and Gr1 surface markers as well as either Annexin V or Ly6G (as previously) and run on the Beckman Coulter FC 500. Purity of sorted cells was consistently greater than 96%.

**Culture of sorted or whole bone marrow cells**

Total bone marrow cells from one FVBN202 mouse were cultured in 10mL dishes with 100ng/mL GM-CSF (Peprotech). Cells were split on day 3 and fresh cytokine added. On day 6 all floating and adherent cells were harvested and used for analysis by flow cytometry. Sorted bone marrow populations (CD11b+Gr1+ and CD11b−Gr1−) were cultured in 6 well plates (1-2×10^5 cells/well) for a total of 6 days. GM-CSF (100ng/mL), VEGF (50ng/mL), or MCP-1 (50ng/mL) were added directly to the culture medium on day 0 (all cytokines/chemokines from Peprotech) or sorted cells were cultured in the bottom of a 6 well dish with a 24mm Transwell insert with a 0.4μm pore on top (Corning Life Sciences). To the Transwell insert, 0.1×10^5 MMC cells were added on day 0. On day 3, cells were split and fresh cytokine was added. Transwell inserts were discarded on day 3 and a fresh insert with 0.1×10^5 MMC cells was added. On day 6, all cells were harvested and aliquoted into tubes for analysis by flow cytometry.

**Isolation of RNA and RT-PCR**

RNA was isolated from magnetically selected or sorted cells via TRizol reagent and was treated with DNase. Total RNA (5μg) was used as a template for reverse transcription (20μL total volume). cDNA was used in a PCR master mix containing 1X PCR buffer, 1.5mM MgCl₂, 2.5U Taq polymerase, and 1μM gene-specific primers: β-actin [5’-
GTGGGCCGCTCTAGGCACCAA-3’ (sense) and 5’-
CTCTTTGATGTACGCACGATTTTC-3’ (anti-sense)]; GAPDH [5’-
ACCACAGTCCATGCCATCAC-3’ (sense) and 5’-TCCACCACCCTGGTGTGTA-3’
(anti-sense)]; TGF-β [5’-AAGACCATCGACATGGAGC-3’ (sense) and 5’-
TGTCACAAGAGCAGTGAGCG-3’ (anti-sense)]; IL-10 [5’-
ATGCAGGACTTTAAGGGTTACTTGGTTT-3’ (sense) and 5’-
ATTTGAGAGAGGTACAAACGAGGTTT-3’ (anti-sense)]; and IDO [5’-
GTACATCCACCATGGCGTATG-3’ (sense) and 5’-GCTTTCTCAAGTCTTCCATTG-3’
(anti-sense)]. PCR conditions were as follows: TGF-β: 94ºC 5 min, 94ºC 1 min, 58ºC 1
min, 72ºC 2 min (42 cycles) followed by a 7 min extension at 72ºC; IL-10: 94ºC 5 min,
94ºC 30 s, 60ºC 30 s, 72ºC 1 min (35 cycles) followed by a 7 min extension at 72ºC; IDO:
94ºC 3 min, 94ºC 30 s, 60ºC 1 min, 72ºC 2 min (40 cycles) followed by a 5 min extension
at 72ºC. Amplified fragments were visualized by ethidium bromide staining of the agarose
gel and photography under UV light in Gel Doc 2000™ (BioRad). β-actin or GAPDH
were used as internal controls.

In vivo treatments with GM-CSF, IL-6, VEGF and MCP-1

Recombinant GM-CSF (500ng), GM-CSF+IL-6 (500ng and 120 units, respectively),
VEGF (1.5µg), or MCP-1 (2.5µg) or PBS was injected i.d. into FVB mice once per day for
3 consecutive days. Mice were sacrificed on day 4 and spleens, bone marrow, and blood
analyzed by flow cytometry for the expression of CD11b and Gr1. GM-CSF (2.5µg) was
administered i.d. for 2 consecutive days and mice were sacrificed on day 3 for analysis of
the spleen, bone marrow, and blood, as above.

*Tumor re-challenge studies:*

FVBN202 mice that rejected initial MMC challenge were challenged again with 3-5X10^6
MMC cells (as indicated) i.d. on the contralateral side. Subsequent challenges were done
i.d. on the side contralateral to the first re-challenge.

*Statistical Analyses:*

Analyses were done using the students, one-tailed, unpaired T-Test. Significant values are
less than or equal to 0.05.
Chapter 1

I. Study Rationale

Our lab has had a long standing interest in developing an effective means of treating neu positive mammary tumors with adoptively transferred T cells. The FVBN202 mouse model is a clinically relevant means by which to investigate adoptive immunotherapy (AIT) because of their immunological tolerance to the neu oncoprotein. Despite this tolerance, it has previously been shown that FVBN202 mice develop atypical hyperplasia of the mammary glands preceding spontaneous tumor development (25) and that this increase in mammary epithelium provides a “danger signal” that results in antibody responses against the neu ECD as well as IFN-γ secretion in response to the neu protein in FVBN202 mice (221). This break in self tolerance, however, was not successful in inhibiting subsequent tumor challenges with neu+ MMC cells, since we have shown that both a non-self entity and a danger signal are needed to mount effective anti-tumor immune responses in these mice (221). Additionally, atypical hyperplasia generated an immunosuppressive microenvironment characterized by the increased expression of IL-10 and the IL-10 receptor, as well as suppressor of cytokine signaling (SOCS)-1 and SOCS-3 (222), which may lead to increased MDSC. We have observed elevated levels of MDSC in FVBN202 mice during the premalignant stage and have correlated this with mammary
hyperplasia and the failure of the pre-existing immune response to prevent spontaneous tumor development (172, 221).

Previous attempts in our lab to use adoptively transferred T cells to cure neu+ tumors in FVBN202 mice have proven unsuccessful, despite the fact that T cells were activated and expanded from FVB mice that had successfully rejected a neu+ tumor challenge. Efforts to identify reasons why expanded T cells from FVB mice fail to reject tumors in FVBN202 mice lead us to the observation that tumor-bearing FVBN202 mice exhibited large increases in their granulocytes. Previous finding in a transgenic mouse model similar to ours that expresses the activated rat neu oncogene have revealed that increases in spontaneous mammary tumor development coincided with increases in CD11b+Gr1+ MDSC (181), which lead us to determine the prevalence of these cells in our model and in response to MMC tumor challenge. The increase in granulocytes previously observed was, in fact, because of large increases in CD11b+Gr1+ cells. A subsequent finding showed that the transfer of T cells into chronically inflamed environments characterized by MDSC resulted in the suppression of those T cells via TCR downregulation (182). The increase in MDSC in tumor-bearing FVBN202 mice, combined with the fact that these mice also exhibit atypical hyperplasia of the mammary glands and elevated MDSC prior to spontaneous mammary development led us to hypothesize that the failure of adoptively transferred T cells in FVBN202 mice is a result of the presence of elevated levels of MDSC. We further hypothesized that the attenuation of these cells or the ability to overcome/circumvent these cells in vivo would allow adoptively transferred T cells to mediate tumor regression.
II. Results

FVBN202 Mice Bearing Neu Positive MMC Tumors Accumulate Large Numbers of CD11b+Gr1+ Cells in their Spleens, Bone Marrow, and Blood

In the FVBN202 mouse model of spontaneously arising mammary carcinomas, we have observed a large increase in cells of the granulocyte region in mice bearing neu-expressing MMC tumors. This increase in the overall percentage of granulocytes has been observed in the spleen, bone marrow, and blood of these mice (Fig. 2). Representative dot plots show that the granulocyte region in the spleens of tumor-bearing mice increases four-fold from 6% to 24%, while also increasing 2.7-fold from 22% to 59% in the bone marrow and 4.3-fold from 7% to 30% in the blood (Fig. 2). Analysis of the absolute numbers of granulocytes present in the spleen and the bone marrow of the tibias and femurs shows a statistically significant increase in granulocytes in MMC tumor-bearing FVBN202 mice over tumor-free FVBN202 mice (Fig. 3). In the spleen, tumor-bearing mice showed an average of 6.56X10⁷ granulocytes, whereas tumor-free mice had an average of 1.11X10⁷ granulocytes (P=0.004, Fig. 3A). Similarly, the number of granulocytes present in the bone marrow recovered from the tibias and femurs of tumor-bearing animals averaged 1.95X10⁷ whereas the average number of granulocytes in the bone marrow recovered from the tibias and femurs of tumor-free mice was 7.2X10⁶ (P=0.003, Fig. 3B). There is no such influx in cells of the lymphocyte region, as shown in Figure 4, the increased granulocytes cause a significant decrease in the overall percentage of cells in the
Figure 2: Accumulation of granulocytes in the spleen, bone marrow, and blood of FVPN202 mice bearing MMC tumors as compared to tumor free FVPN202 mice. Flow cytometry analysis of total splenocytes, bone marrow cells, and peripheral blood cells isolated from tumor bearing or tumor free female FVPN202 mice. Granulocytes were identified according to their size (forward scatter-FS) and granularity (side scatter-SS).

Percentages represent the percent of granulocytes out of the total cells. Data are representative of 4-6 experiments.
Figure 3: FVBN202 mice bearing MMC tumors have significantly increased numbers of MDSC in both their spleens and bone marrow.

Flow cytometry forward scatter vs side scatter analysis was used to identify the granulocyte region within total spleen cells (A), or bone marrow cells that were isolated from the femurs and tibia (B) of FVBN202 tumor bearing and tumor free mice. The absolute number of granulocytes was then calculated using the percentage and the total cell number, as determined by trypan blue exclusion cell counts using a hemocytometer. Data are averages of 3-4 mice per group. Representative plots of the granulocyte regions from tumor-bearing vs. tumor-free spleens and bone marrow can be found in Figure 1. All tumor bearing mice had tumors between 1100 and 2000mm³ and were 8-18 weeks of age.
A. Absolute number of granulocytes in the spleen

B. Number of granulocytes from the bone marrow of tibias and femurs
lymphocyte region of the spleen (39.2% in tumor-bearing vs. 59.3% in tumor free, P=0.0377), although this is not accompanied by a significant decrease in the absolute number of these cells (11.3X10^7 in tumor-bearing vs. 8.99X10^7 in tumor-free) indicating that the presence of the granulocytes does not inhibit the persistence of cells in the lymphocyte region in the periphery, but rather causes a shift in the lymphocyte to granulocyte ratio (Fig. 4A,C). In the bone marrow, however, both the percentage and the absolute number of cells in the lymphocyte region recovered from the tibias and femurs are significantly decreased, with tumor-bearing mice having only 2.4X10^6 cells in the lymphocyte region whereas tumor free-mice averaged 9.4X10^6 cells in the lymphocyte region (Fig. 4D, P=0.001). Furthermore, the percentage of cells in the lymphocyte region decreased by 4.9-fold, from 43.7% in tumor-free bone marrow, to 9% in tumor-bearing bone marrow (Fig. 4B,D P=0.001). This indicates that the increased number of granulocytes may out-compete cells in the lymphocyte region for available cytokines. Further investigation of the granulocyte region in these mice reveals the presence of cells staining double positive for the integrin CD11b and the glycosylphosphatidylinositol-linked myeloid differentiation protein Gr1. We have found in every compartment in vivo, that the presence of CD11b+Gr1+ cells increases in MMC tumor-bearing FVBN202 mice as compared to naïve FVBN202 mice. We have found these cells to increase both as a percentage from within the granulocyte region, as well as a percentage of the overall spleen (Fig. 5). In the spleen, the total percentage of CD11b+Gr1+ cells increases from 3% to 34% in naïve versus tumor-bearing animals and from 8% to 92% in comparing naïve versus tumor-bearing granulocytes, respectively. A similar observation was made in the
bone marrow, where the total percentage of CD11b+Gr1+ cells increased from 28% in naïve mice to 76% in tumor-bearing mice and from 77% of the granulocytes in naïve mice to 91% of the granulocytes in tumor-bearing mice (Fig. 5). Likewise, large increases in double positive cells are observed in the blood (4% of the total peripheral blood leukocytes, or PBL, in tumor-free versus 34% of the total PBL in tumor-bearing). The percentage of double positive cells comprising the granulocyte region of the blood increases to 84% in tumor-bearing mice from 54% found in tumor-free mice. A somewhat smaller population of double positive cells (9%) was also observed at the tumor site itself when MMC tumors were excised and subjected to flow cytometry (Fig. 5).
Figure 4: The presence of elevated MDSC in tumor bearing FVBN202 mice results in a drastic reduction of lymphocyte precursors in the bone marrow.

Flow cytometry forward scatter vs side scatter analysis was used to identify the lymphocyte region within total spleen cells (A), or bone marrow cells (B) from FVBN202 tumor bearing and tumor free mice. Figure 1 shows percentages spleen and bone marrow cells comprised of granulocytes in these mice. The absolute number of lymphocytes was then calculated using the percentage and the total cell number from the spleen (C) or number of bone marrow cells recovered from the tibias and femurs (D) as determined by trypan blue exclusion cell counts using a hemocytometer. Data are averages of 3-4 mice per group and mice were 8-18 weeks of age.
Figure 5: Accumulation of CD11b+Gr1+ cells in FVBN202 MMC tumor-bearing mice as compared to FVBN202 tumor free mice

Flow cytometry was performed using monoclonal antibodies against the CD11b+ and Gr1+ surface antigens in order to identify MDSC. The percentage of CD11b+Gr1+ MDSC is shown both out of the total cell population (Left column) and out of the granulocyte region (right column) for splenocytes, bone marrow, and peripheral blood. Additionally, CD11b+Gr1+ cells were identified at the tumor site as well. Data are representative of 4-6 experiments.
Total | Granulocyte Region
--- | ---
Naive FVB/N202 splenocytes
MMC tumor-bearing FVB/N202 splenocytes
Naive FVB/N202 bone marrow
MMC tumor-bearing FVB/N202 bone marrow
FVB/N202 TF blood
FVB/N202 MMC TB blood
MMC tumor site in FVB/N202 mouse
**Bryostatin-1/Ionomycin (B/I) stimulation followed by IL-2 expansion generates highly activated neu-specific effector T cells**

Since parental FVB mice recognize the rat neu protein as a foreign antigen and are subsequently able to reject MMC, whereas FVBN202 mice often tolerate neu protein and are unable to reject MMC, FVB mice were used as donors for AIT transfers into FVBN202 recipients in these studies. Since B/I selectively activates effector T cells regardless of their antigen specificity (139), we sensitized FVB mice with MMC cells in order to increase the pool of neu-specific effector T cells. We first compared the populations of CD4+ and CD8+ T cells from these donors immediately after harvest, after activation with B/I, and after a 7-day expansion with IL-2. Representative data from duplicate experiments are presented in Figure 6. FVB donor splenocytes contained 35% and 9% CD4+ and CD8+ T cells, respectively. These populations were similar immediately after B/I activation (27% and 12% CD4+ and CD8+, respectively) but were greatly increased after 7 days of culture with IL-2 (Fig. 6A, 55% and 32% CD4+ and CD8+, respectively). Furthermore, IL-2 treatment increased the absolute number of viable T cells by 9.5-fold over the cell number that was cultured after B/I expansion (Fig. 6B). The Annexin V+ CD4+ population remained nearly constant during *ex vivo* expansion, starting at 20% on day 0 compared with 24% after B/I activation and cytokine treatment (Fig. 6C). The CD8+ T cells, however, showed a marked increase in Annexin V+ staining from day 0 to day 7, with the fresh splenocytes being only 13% Annexin V+, while the post-B/I and post-cytokine values were 23% and 54%, respectively (Fig. 6C).
Figure 6: Flow cytometry analysis of T cell viability and phenotypes before and after activation with B/I.

(A) Analysis of the total percentage of cells comprising the CD4+ and CD8+ populations before and after B/I activation of donor splenocytes. (B) Flow cytometry staining for apoptotic (Annexin V positive) CD4 and CD8 T cells. Plots are gated on either CD4+ or CD8+ cells from within the lymphocyte region. (C) Analysis of Central memory (CD62L+CD44+) and effector (CD62L-CD44+) T cells from the CD4+ and CD8+ compartments of the lymphocyte region. (D) Analysis of activation markers CD25 and CD69. Plots are gated on either CD4+ or CD8+ cells from within the lymphocyte region. Data are representative of 2 separate experiments.
In order to determine T cell phenotypes, flow cytometry was performed for memory T cells (CD44+CD62L+) and effector T cells (CD44+CD62L-), as well as for the activation marker CD25, and the very early activation marker CD69, in both the CD4+ and CD8+ populations. As expected, B/I activation greatly increased the effector phenotype in the CD4+ population from 16% to 79% and in the CD8+ population from 5% to 66% while naïve (CD44-CD62L+) and memory (CD44+CD62L+) CD4+ and CD8+ T cells were greatly decreased (Fig. 6D). After IL-2-induced expansion, both CD4+ and CD8+ T cells showed a marked increase in CD44+CD62L+ memory T cells (74% and 58% in the CD4+ and CD8+ compartments, respectively), while maintaining increased levels of CD44+CD62L- effector T cells compared to pre-B/I treatment (16% vs. 22% and 5% vs. 38%, respectively). Additionally, the expression of CD25 on CD4+ and CD8+ T cells was markedly increased to over 80% in both cases after B/I activation, and increased to over 90% in both populations after IL-2 expansion (Fig. 6E). The very early activation marker, CD69, was greatly increased from about 0.1-0.2% in fresh splenocytes to 87% in CD4+ T cells and 93% in CD8+ T cells after B/I activation. This value, however, dropped again after a 7-day culture with IL-2, with 0.8% CD69 expression in CD4+ T cells, and, notably, 3% remaining in the CD8+ T cells. However, most cells retained a late effector phenotype (CD44+CD69-) on day 7 (Fig. 6F).

To confirm anti-tumor efficacy in vitro, T cells derived from MMC sensitized FVB mice prior to (Pre-B/I) or after a 7-day ex vivo expansion (Post-Cytokine) were co-cultured with MMC target cells (E:T ratio of 10:1) for 48 hours followed by staining with antibodies directed towards neu, Annexin V, and PI. Control wells were seeded with MMC
in the absence of T cells (No Treatment) (Fig. 7). Gating on neu positive cells and analyzing the percentages of Annexin V and PI positive cells allowed for the determination of specific killing of neu positive MMC cells by T cells. The viability of MMC in the absence of T cells was 86% (Annexin V and PI negative) while it dropped to 44% in the presence of the freshly isolated T cells (Pre-B/I). Viability of MMC was further decreased to 27% when cultured with B/I-activated, IL-2-expanded T cells (Post-Cytokine) (Fig. 7A). Absolute numbers of viable MMC also reflect the increased anti-tumor efficacy of B/I-activated, IL-2-expanded T cells compared to freshly isolated T cells ($P=0.026$) (Fig. 7B). No killing was detected against the neu negative tumor variant, as determined by trypan blue exclusion (data not shown).
Figure 7: Anti-tumor efficacy of neu-specific effector T cells before and after activation
with B/T and expansion with IL-2

(A) Annexin V and PI analyses of gated neu positive MMC after 48 hours of culture alone
(top), or with neu-specific lymphocytes before (middle) or after (bottom) B/T activation and
7 days expansion in the presence of IL-2. Representative data of gated neu positive cells are
shown from two independent experiments. (B) Cell counts using trypan blue exclusion for
quantification of the total number of viable MMC. Data are averages of 2-4 experiments +/-
SEM.
A. 

- MMC
- Pre-Ed
- Post-Cytokine

B. 

Cytotoxicity of donor T cells before B/I stimulation and after 7 days of IL-2 expansion.

- No treatment
- Pre-B/I
- Post Cytokine

Cell number X10^6

P = 0.0001
P = 0.026
**In vivo Depletion of MDSC is Required for the Efficacy of IL-2 Expanded Adoptively Transferred T cells**

We next wanted to determine if transfer of these IL-2 expanded, highly cytotoxic T cells could result in tumor inhibition in FVBN202 mice. To test this, FVB mice were inoculated i.d. with $5 \times 10^6$ MMC cells. Spleens were harvested after the mice had fully rejected the tumors and cells were activated with B/I and subsequently expanded in the presence of IL-2 as before. Twenty-four hours prior to the transfer of expanded T cells, FVBN202 mice were treated with 100 mg/kg of cyclophosphamide (CYP) in order to create a lymphopenic environment, which has been shown to be beneficial for the expansion of transferred T cells (128, 129). After 24 hours, mice were challenged i.d. with $5 \times 10^6$ MMC cells and $7 \times 10^6$ expanded T cells were transferred into each FVBN202 mouse that was to receive adoptive transfer via tail vein injection. Since FVBN202 mice bearing MMC tumors exhibit a pronounced accumulation of MDSC in vivo, (Fig. 5), we hypothesized that adoptively transferred T cells may be prevented from working in such an immunosuppressive environment. Therefore, one group of mice that received MMC challenge and adoptive transfer of T cells also received i.p. injections of an anti-Gr1 antibody (300µg per injection starting on day 6 and every three days thereafter for a total of 4 injections). Tumor growth measurements in Figure 8 show that the tumor volumes did not differ significantly between the control group (CYP +MMC) and the group that also received adoptively transferred T cells. Strikingly, there was significant tumor inhibition in the group treated with anti-Gr1 antibody compared to the control group at weeks 6 (P=0.018) and 7 (P=0.032), with the mice receiving Gr1 depletions having an average
tumor volume of only 26.9 mm$^3$ on week 6 as compared to 588.9 mm$^3$ in the control group, and 32.7 mm$^3$ on week 7 as compared to 1121.4 mm$^3$ in the control group. Therefore, adoptive transfer of anti-neu T cells expanded with IL-2 and highly effective \textit{in vitro} is not effective unless combine with the depletion of endogenous MDSC. To eliminate the possibility that the depletion of endogenous MDSC may, on its own, result in tumor rejection by these mice by allowing them to break tolerance to MMC tumors, FVBN202 mice were inoculated with $5 \times 10^6$ MMC cells intradermally (i.d.) and were treated with anti-Gr1 antibody starting 6 days after tumor inoculation and continuing every three days for a total of 3 injections. As shown in Figure 9, all mice in this group had large tumors by week 3, therefore confirming that MDSC depletion alone cannot cause the rejection of tumors to which an individual is tolerant.
Figure 8: Adoptive immunotherapy using IL-2 expanded T cells is not advantageous unless coupled with the depletion of MDSC.

FVBN202 mice were pretreated 24 hours prior with 100mg/kg CYP. On Day 0, 70×10^6 T cells from FVB donor mice that had been expanded in IL-2 for 7 days were injected into the tail veins of FVBN202 recipient mice, which were also challenged with 5×10^6 MMC’s intradermally the same day. One group of mice did not receive any T cell transfer and served as the control group (circles). Another group got T cell transfer alone (triangles). The last group got T cell transfer, and, starting on day 6, received 300μg of LEAF purified anti-Grl via i.p. injection every 3 days of a total of 4 injections. Tumor growth was measured by digital caliper and the volumes calculated as stated in the materials and methods. There were 3 mice per group.
AIT using IL-2 expanded T cells with and without Gr1+ depletions

Tumor volume (mm³)

Weeks after inoculation

- ▲ Cyp+MMC+IL-2 expanded T cells
- ● Cyp+MMC
- ■ Cyp+MMC+IL-2 expanded T cells + MDSC depletion

P = 0.018
P = 0.032
Figure 9: The in vivo depletion of Gr1+ cells alone does not inhibit tumor growth in FVBN202 mice.

3 FVBN202 mice that were 2 months of age were inoculated intradermally with 5X10⁴ MMC cells and were given 250µg of anti-Gr1 antibody starting on day 6 after inoculation and continuing every 3 days for a total of 3 injections. Mice were not treated with Cyp 24 hours prior to MMC inoculation.
Mice receiving MMC challenge and MDSC depletion alone

Tumor volume [mm$^3$]

Weeks after inoculation
The Efficacy of in vivo MDSC depletions can be directly correlated with the effectiveness of the anti-tumor response

In order to make sure that the anti-Gr1 antibody injections were effectively removing MDSC from MMC-challenged FVB/N202 mice in vivo, flow cytometry of the peripheral blood was done regularly. High levels of CD11b+Gr1+ cells were observed in control mice and mice receiving adoptive transfer without MDSC depletion. As expected, these levels were consistent with those seen previously in tumor bearing mice (representative plots from tumor bearing mice shown in Fig. 5). However, flow cytometry of the peripheral blood from mice receiving anti-Gr1 antibody on day 21 showed that this depletion was almost completely effective in 2 out of three mice (Fig. 10A), with the granulocyte regions almost completely ablated in both cases (1% remaining in mouse 1 and 2% remaining in mouse 2). In contrast, mouse 3 was less responsive to treatment with anti-Gr1 antibody and maintained 14% granulocytes on day 21 (Fig. 10A). The level of surface expression of the Gr1 molecule is depicted in Fig. 10B. The mean fluorescent intensity (MFI) of the Gr1 molecule in mouse 3 is 102.6, whereas the MFI of the Gr1 molecule in mouse 1 and mouse 2 are 1.4 and 1.5, respectively. Interestingly, the efficacy of Gr1 depletions seems to correlate positively with tumor rejection, with the tumor volumes of mouse 1 and mouse 2 on day 21 being 8.9 mm$^3$ and 0 mm$^3$, respectively, whereas the tumor volume of mouse 3 on day 21 was 283.19 mm$^3$ (Fig. 10B). This trend is seen in Figure 11, where mouse 1 and mouse both eventually rejected tumors, while mouse 3 had a tumor measuring 545.5 mm$^3$ by the end of week 4. Not only did the depletion of MDSC in vivo significantly aid in tumor rejection, but it also resulted in an antibody
Figure 10: Levels of MDSC in the peripheral blood of mice receiving anti-Gr1 antibody

(A) Forward scatter vs side scatter flow cytometry analysis of the peripheral blood on day 21 of 3 mice receiving anti-Gr1 antibody. Percentages represent the percent of the whole PBL that is comprised either of lymphocytes or granulocytes in each mouse. (B) Overlay plot shows the expression of the Gr1 surface antigen in the granulocyte region from the peripheral blood of the 3 mice receiving injection of anti-Gr1 antibody. Two of the 3 mice were completely depleted and showed no residual Gr1 expression. The third mouse was not completely depleted of Gr1+ cells in the peripheral blood. Blood was taken on day 21 after MMC injection and stained using an anti-mouse Gr1 antibody and measured by flow cytometry.
% Lymphocytes | 17 | 16 | 17
% Granulocytes | 1  | 2  | 14

E.

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Figure 11: Tumor growth is correlated with the persistence of Gr1− cells *in vivo*

Plot shows the tumor volumes of the three individual mice that received adoptively transferred T cells and Gr1+ depletions. Mouse #3, is the mouse previously shown have maintained Gr1+ cells in the peripheral blood, while the other 2 mice were completely depleted.
Tumor volumes of individual mice receiving IL-2 expanded T cells and Gr1+ depletions.

- Mouse #1
- Mouse #2
- Mouse #3

Weeks after inoculation

Tumor volume (mm³)
Figure 12: Depletion of Gr1+ cells \textit{in vivo} restores an anti-neu antibody response in tolerant FVB/N202 mice

Blood was drawn from the retro-orbital sinus 21 days after MMC inoculation and serum collected after centrifugation. Serial dilutions of the serum were added to 96 well plates that had previously been coated with the extracellular domain of HER2/neu. Data are the average O.D. readings of the 3 mice from each group, done in duplicate.
Antibody responses to the extracellular domain of HER2/neu

- ▲ Cyp+MMC+IL-2 expanded T cells
- ○ Cyp+MMC
- ▼ Cyp+MMC+IL-2 expanded T cells + MDSC depletion
response against the extracellular domain (ECD) of neu. Figure 12 shows that only mice receiving adoptive transfer along with MDSC depletion displayed an antibody response, whereas control mice and mice receiving adoptive transfer alone showed no antibody response, as is typical in this tolerant model. Interestingly, the strength of anti-neu antibody responses seems to also correlate with the efficacy of MDSC depletion. As shown in Figure 13, mouse 1 and mouse 2, which showed the highest degree of depletion, had noticeably stronger antibody responses than mouse 3, which was not efficiently depleted of MDSC \textit{in vivo}.

In order to determine if the reason for tumor regression in mice receiving adoptive transfer of neu specific T cells with along with MDSC depletion was a greater persistence of activated T cells, mice were sacrificed 47-51 days after tumor inoculation and their spleens were analyzed by flow cytometry for the presence of activated T cells. Staining for CD25 expression on CD4+ and CD8+ T cells showed that in both cases (CD4 and CD8), mice receiving adoptive transfer alone had the highest percentage of activated T cells (9.2% CD25+CD4+ T cells and 4.1% CD25+CD8+ T cells). In contrast, control mice and mice receiving adoptive transfer along with MDSC depletion had similarly low levels of CD25+ T cells (4.6% CD25+CD4+ in control mice and 5.3% CD25+CD4+ in mice receiving MDSC depletion; 1.8% CD25+CD8+ in control mice and 1.7% CD25+CD8+ in mice receiving MDSC depletion, Fig. 14). Therefore, the presence of activated T cells alone is not sufficient for the rejection of MMC tumors, and in the absence of suppressive MDSC, mice can remain tumor free even in the absence of high levels of activated T cells.
Figure 13: Lack of antibody response is correlated with the persistence of Gr1+ cells \textit{in vivo}.

Three individual mice that received adoptively transferred T cells along with anti-Gr1 antibody injections. Mouse #3 showed persistence of Gr1+ cells \textit{in vivo}, while mice #1 and #2 were completely depleted. Data are the averages O.D.'s of duplicate wells of a serum ELISA against the extracellular domain of HER2/neu.
Antibody responses against the extracellular domain of HER2/neu in individual mice receiving IL-2 expanded T cells and Gr1+ depletions.
Figure 14: Flow cytometry of splenocytes from FVBN202 recipient mice at the termination of the experiment

Splenocytes from mice from each of the three treatment groups were harvested and using monoclonal antibodies against CD4, CD8, and CD25. Cells were fixed and flow cytometry was run within 24 hours. Plots are gated on either CD4+ or CD8+ populations from within the lymphocyte region of the spleen. MMC+Cyp and ATT alone mice were killed 47 days after inoculation, Gr1 depletion mice were killed 51 days after inoculation.
T Cell Expansion Using Alternating Gamma Chain Cytokines Generates More Potent Anti-Tumor T cells with Greater Levels of Expansion.

In an effort to generate T cells that may be more potent in the face of high levels of MDSC, we sought to devise a superior method of T cell expansion. We had previously attempted T cell expansion in the presence of IL-7 and IL-15, and noticed that although viability was improved over that seen in expansion with IL-2 alone (10% Annexin V+ CD4+ T cells after IL-7/15 as compared to 24% Annexin V+ CD4+ T cells after IL-2 expansion and 13% Annexin V+ CD8+ T cells after IL-7/15 expansion as compared to 54% Annexin V+ CD8+ T cells after IL-2 expansion), expansion levels were not great enough to generate enough T cells for injection on Day 7 (Figs. 6C,15B, 16). Also noteworthy is a severe reduction in the levels of activated T cells on day 7 after expansion with IL-7/15 (45% CD25+CD4+ and 9% CD25+CD8+, Fig. 15D) as compared to expansion with IL-2 (99% CD25+CD4+ and 92% CD25+CD8+, Fig. 6E). In order to optimize the benefits of both types of expansion, we decided to use treatment with IL-7/15 (10ng/ml) on days 1, 3, and 5, with a one time “pulse” of IL-2 (40 U/ml) on day 2. We refer to this as “alternating gamma chain cytokine” treatment. This type of expansion increased the percentage of CD8+ T cells (47% in Fig. 15A vs. 32% in Fig. 6A) and showed an 11-fold expansion in overall viable T cell number as compared to a 9.5-fold expansion with IL-2 treatment (Fig. 16). Alternating gamma chain cytokines also greatly enhanced the viability of T cells on day 7 of culture when compared to expansion with IL-2 alone (Fig. 15B shows 13% Annexin V+ CD4+ T cells and 11% Annexin V+ CD8+ T cells compared with 24% Annexin V+ CD4+ T cells and 54% Annexin V+ CD8+ T cells
Figure 15: Flow cytometry analysis of T cells after 7 days of cytokine expansion using either IL-2, IL-7 and IL-15, or a combination of all three, termed “alternating” cytokine expansion.

(A) Analysis of the total percentage of CD4+ and CD8+ cells present at the end of each type of expansion period. (B) Analysis of the viability of CD4+ and CD8+ T cells at the end of each expansion period. Plots are gated on either CD4+ or CD8+ cells from within the lymphocyte region. (C) Analysis of central memory (CD62Lmid/CD44low), effector memory (CD62Llow/CD44high) and effector (CD62L-CD44+) T cells from within the CD4+ and CD8+ compartments of the lymphocyte region after 7 days of expansion with each type of cytokine treatment. (D) Analysis of the expression of the activation markers CD25 and CD69, gated on either CD4+ or CD8+ T cells from within the lymphocyte region.
in Fig. 6C). Of note, there were more CD44+CD62L− effector T cells in both the CD4+ and CD8+ compartments after expansion using alternating gamma chain cytokines as compared to expansion with IL-2 alone (33% vs. 22% CD4+ T cells and 48% vs. 38% CD8+ T cells, Figs. 15C and 6D). Like expansion in IL-2 alone, expansion with alternating gamma chain cytokines generated only CD62L\textsuperscript{low} effector memory T cells whereas expansion with IL-7 and IL-15, generated a population of CD62L\textsuperscript{high} central memory T cells. It has previously been shown that the adoptive transfer of B/I activated CD62L\textsuperscript{low} cells was able to mediate complete regression of the 4T07 murine mammary tumor while adoptive transfer of the CD62L\textsuperscript{high} fraction resulted in only a 17% regression of tumors (137). While the percentage of CD4+CD25+ T cells and CD4+CD69+ T cells remained unchanged, CD8+CD25+ T cells decreased from 92% to 69% and CD8+CD69+ T cells decreased from 3% to 0.3% in comparing the IL-2-expanded T cells with alternating cytokine-expanded T cells, respectively (Figs. 6 E,F and 15 D,E). Although levels of CD25+ CD8+ T cells dropped in cells expanded with alternating cytokines as compared to cells expanded in IL-2 alone, levels of activated T cells were greatly increased in alternating cytokine-expanded cells as compared to IL-7/15 expanded cells (Fig. 15 D). Expression of the IL-7Rα (CD127), necessary for IL-7 to exert its effects in maintaining pools of memory T cells (223, 224), was measured after each type of cytokine expansion, and also on day 3 of alternating gamma chain cytokine expansion. CD127 is expressed on only 9% of CD4+ cells and 15% of CD8+ cells after expansion in IL-2 (Fig 17). However, after expansion in IL-7/15, expression of CD127 is negligible (0.08% on CD4+ cells and 0.03% on CD8+ cells) indicating the inability of these cells to expand is because of the
Figure 16: Fold expansion after cytokine treatments

Comparison of the fold expansion achieved after each of the three types of cytokine expansion. Data shows the absolute number of viable cells at the end of the expansion period divided by the absolute number of viable cells that were present after activation with B/I. Number of viable cells was determined by trypan blue exclusion. Data are representative of 2-5 separate experiments.
Fold-expansion of FVB donor cells as a result of different cytokine treatments

Fold-Expansion

0  5  10  15

Type of cytokine treatment

Alternating  IL-2  IL-2/5
downregulation of at least one of the necessary cytokine receptors. Interestingly, on day 3 of the alternating gamma chain cytokine expansion regimen, expression of CD127 is present on only 2% of CD4+ cells and 3% of CD8+ cells. However, these levels rise and are greatly elevated by day 7, with 21% of CD4+ cells and 18% of CD8+ cells expressing CD127 (Fig. 17). This is interesting since IL-2 has been shown to promote expression of CD127; however IL-2 expanded cells, and alternating cytokine expanded cells after IL-2 pulse, show low levels of CD127, which increase without additional exogenous IL-2 (223). However, it has been shown in humans that CD8+ T cells with low IL-7Rα expression have increased levels of the IL-2/IL-15Rβ chain and respond to IL-15 with increased proliferation (225). Therefore the low expression of CD127 in Figure 17 could be indicative of cells with increased responsiveness to IL-15, which has been shown to be important for the maintenance and function of memory CD8+ T cells (148). Since the CD127 receptor is necessary for the homeostatic expansion of cells in vivo and for maintenance, particularly of CD8+ memory T cells, it is imperative to expand the cells in such a way that facilitates the continued expression of this receptor, as opposed to expansion methods that may cause receptor downregulation by negative feedback mechanisms (226). Another concern in the generation of T cells in vitro has been the induction of regulatory T cells, particularly in response to IL-2 expansion. Figure 18 shows flow cytometry staining for regulatory T cells both before and after B/I stimulation and after each type of cytokine expansion. Plots are gated on CD4+CD25+ cells so as to discern the percentage of FoxP3+ cells amongst activated CD4+ cells. Very low expression of FoxP3 before stimulation with B/I indicates that 5.1% of CD4+CD25+ T cells also
Figure 17: CD127 expression on FVB donor lymphocytes

Flow cytometry was done to analyze the expression of the IL-7 receptor alpha chain after B/I activation and various cytokine treatments. (A) CD127 staining on day 7 after expansion with IL-2 alone. (B) CD127 staining on day 7 after expansion with IL-7 and IL-15 alone. (C) CD127 staining on day 3 during expansion using only IL-7 and IL-15. (D) CD127 staining on Day 7 after expansion using the alternating cytokine regimen. All Plots are gated on CD4+ or CD8+ cells from within the lymphocyte population.
express FoxP3. This number drops to 1.54% after activation (1.54%), but increases substantially after IL-2 expansion to 10.03%. Conversely, Tregs constituted only 4.08% of activated T cells after expansion in IL-7/15, whereas expansion in alternating gamma chain cytokines resulted in 12.0% of CD4+CD25+ cells containing FoxP3. Given the fact that Figure 16 shows that T cell expansion in vitro was the greatest using alternating gamma chain cytokines (which had the highest percentage of Foxp3+ cells), and that expansion using IL-7/15 produced the smallest number of cells despite having the lowest percentage of Foxp3+ cells, we have determined that CD4+CD25+FoxP3+ cells do not necessarily constitute a regulatory phenotype in our model and are not capable of inhibiting cytokine-driven expansion of activated T cells. Additionally, transferred CD4+CD25+FoxP3+ T cells did not inhibit effective anti-tumor responses in the absence of MDSC (Fig. 8). The cytotoxic effect of T cells against MMC, in vitro, was also greater using alternating gamma chain cytokines compared to that using IL-2 (14% viable MMC in Fig. 19A vs. 27% viable MMC in Fig. 7A). Absolute numbers of viable MMC also reflects a slight increase in anti-tumor efficacy of B/I-activated, alternating cytokine-expanded T cells compared to IL-2-expanded T cells (0.4x10^6 in Fig. 19B vs. 0.5x10^6 in Fig. 7B). Cells expanded in alternating cytokines also exhibited a strong IFN-γ response when stimulated with irradiated neu positive MMC (15000 rad), but not with neu-negative ANV cells (P=0.006), thus confirming the neu specificity of these cells (Fig. 19C).
Figure 18: The presence of CD4+CD25+FoxP3+ cells in vitro does not inhibit T cell expansion

Cells taken either directly from FVB donor mice that had been challenged with MMC (Pre-BI), after B/I activation of FVB donor splenocytes (Post-BI), or after expansion with either IL-2 alone, IL-7 and IL-15, or the alternating gamma chain cytokine treatment. Cells were triple stained for CD4, CD25, and intracellular FoxP3. Analyses are gated on CD4+CD25+ cells.
Figure 19: Effector function of expanded T cells against MMC target cells

(A) MMC cells were cultured for 48 hrs with medium (MMC) or in the presence of the neuro-specific lymphocytes activated with B77 and expanded with alternating gamma chain cytokines (T cells expanded with “alternating” cytokines). Annexin V and PI analyses were performed on gated neu positive MMC by flow cytometry. (B) Cell counts using trypan blue exclusion were done for quantification of the total number of viable MMC. (C) IFN-γ secretion by T cells expanded in alternating cytokines and cultured at a 10:1 ratio with irradiated MMC or ANV target cells for 24 hours. Supernatants were collected and stored in the -80°C until used with the mouse IFN-γ ELISA kit (BD Biosciences). Data are the average O.D. (450nm) of duplicates +/- SD.
Adoptive Transfer of T Cell Subsets Expanded with Alternating Gamma Chain Cytokines Inhibits Tumor Growth when Combined with In Vivo MDSC Depletion

We hypothesized that a more robust and highly viable population of anti-tumor T cells expanded in vitro may be able to overcome inhibition by MDSC in tumor-bearing FVBN202 mice. To test this hypothesis, FVB mice were inoculated with MMC (5x10^6 cells/mouse) and donor splenocytes were prepared 21 days after tumor challenge, when animals had rejected MMC. T cells were activated with B/I and were then expanded in the presence of alternating gamma chain cytokines as described above (Fig. 15). All FVBN202 mice were treated with i.p. injection of CYP (100 mg/kg) in order to create lymphopenia. Flow cytometry of the peripheral blood before, and 24 hours after, CYP injection, showed no effect of this drug on the CD11b+Gr1+ population. FVBN202 mice were challenged with MMC (3X10^6) 24 hours after CYP treatment. MMC-challenged mice then received no treatment, the alternating gamma chain cytokine-expanded T cells alone (i.v. injection of 70x10^6 lymphocytes/mouse), or AIT combined with the depletion of MDSC by i.p. injection of 250μg of anti-Gr1 antibody starting 6 days after tumor inoculation and continuing every 3 days for a total of 6 injections, followed by a final injection of 200μg on day 25. Figure 20 shows that AIT alone offered no protection against MMC tumors. However, the in vivo depletion of MDSC improved the efficacy of AIT and caused significant tumor inhibition (P=0.001 for week 4 and P= 0.0003 for week 5). The efficacy of MDSC depletion was above 98% nine days after tumor challenge (data not shown). Flow cytometry analysis of blood collected on day 9 from mice receiving MDSC depletion showed a complete lack of granulocytes, as compared to blood from the same mice taken
Figure 20: Adoptive immunotherapy using T cells expanded in alternating gamma chain cytokines is only effective when combined with Gr1+ depletions in vivo. FVB/N202 recipient mice were pretreated with CYP as before, and were divided into 3 groups, those receiving MMC inoculation alone, those receiving MMC inoculation with adoptively transferred T cells, and those receiving MMC inoculation, adoptively transferred T cells, and 250μg of anti-Gr1 antibody starting on day 6 and continuing every 3 days for a total of 6 injections. Graph represents the averaged tumor volumes of 3-5 mice per group.
AIT with alternating cytokine-expanded T cells

- ▲ Cyp+ MMC+ alternating cytokine expanded T cells
- ● Cyp+ MMC
- ■ Cyp+MMC+alternating cytokine expanded T cells + MDSC depletion

weeks after inoculation

Tumor volume (mm³)
on day 35, 10 days after the last injection of anti-Gr1 antibody (Fig. 21A). Quantitative analysis of CD11bGr1 levels from each group 24 days after the tumor challenge showed that the group receiving Gr1 depletions had a significantly reduced percentage of MDSC (P=0.044 as compared to mice receiving no treatment and P=0.023 as compared to mice receiving AIT alone, Fig. 21B). However, it is noteworthy that 38% of the granulocytes were still CD11b+Gr1+ at this time, a problem that we believe to be caused by slightly increased tumor burden in these mice leading to increased recruitment of MDSC (Fig. 20). Despite the residual MDSC in these mice, we were not able to increase the frequency of antibody injections because of toxicity of the antibody (data not shown).

Since the ECD-specific antibody response is also involved in the protection against neu positive mammary tumors (87, 227-229), we sought to determine whether FVBN202 mice mounted an antibody responses against ECD following AIT. Serum taken from mice receiving adoptive transfer of T cells expanded with alternating gamma chain cytokines with and without the in vivo depletion of MDSC, along with control mice, indicated that only mice that were depleted of Gr1+ cells were able to mount an antibody response against the neu ECD (P=0.006) (Fig. 22).

In order to determine if the depletion of MDSC resulted in an increased frequency of activated or effector T cells, mice were sacrificed 28 days after tumor inoculation and splenocytes were analyzed for the expression of CD44, CD62L, CD25, and CD69 on both CD4+ and CD8+ T cells. As shown in Figure 23A, the percentage of activated, CD4+CD25+ T cells was approximately equal in mice receiving AIT alone versus mice receiving AIT with MDSC depletion (10.9% vs 10.0%). Likewise, the percentage of naïve
Figure 21: The efficacy of Gr1+ depletions is an important factor in the success of adoptive immunotherapy.

(A) Flow cytometry analysis showing forward scatter vs. side scatter of peripheral blood cells taken from mice receiving Gr1 depletions on day 9 and day 35 with a distinct lack of (day 9) and reappearance of (day 35) the granulocyte region. (B) Percentage of CD11b+Gr1+ cells from within the granulocyte region of the peripheral blood as measured by flow cytometry on day 35.
Figure 22: Only mice receiving Gr1+ depletions exhibit an antibody response against the extracellular domain of HER2/neu.

Mean antibody titers of each group previously described from ELISA's done by coating wells with 10μg/mL of HER2/neu ECD and using serum serum taken on day 21 and done in duplicate.
Figure 23: Flow cytometry of T cell phenotypes from the spleens

At the termination of the experiment, 28 days after inoculation, splenocytes were homogenized and analyzed by flow cytometry. (A) staining for CD4 and CD25. Plots are gated on CD4+ lymphocytes. (B) staining for central memory (CD62L+CD44+) and effector (CD62L-CD44+) T cells. Plots are gated on either CD4+ or CD8+ lymphocytes.

(C) Staining for the early activation marker CD69. T cells expressing the very early activation marker CD69 and CD44. Plots are gated on either CD4+ or CD8+ lymphocytes.
(CD62L+CD44-), effector memory (CD62L<sub>low</sub>CD44+), and effector (CD62L-CD44+) CD4+ T cells were similar between the two groups (Fig. 23B). However, in the CD8+ population, it is noteworthy that the levels of naïve T cells were actually higher in the group receiving MDSC depletion (35.5% in AIT alone mice vs. 42.6% in MDSC depleted mice), while levels of effector memory CD8+ T cells were reduced (49.4% central memory in the AIT alone group vs. 35.5% in the MDSC depleted group). Levels of CD8+CD44+ T cells were similar between the 2 groups (12.1% in the AIT alone group vs. 10.4% in the MDSC depleted group, Fig. 23B). We also wanted to look for expression of the early activation marker, CD69. As shown in Figure 23C, CD69 expression on CD4+ and on CD8+ cells from both groups is similar. To determine if the overall proportion of lymphocytes in animals receiving MDSC depletion may be increased, therefore aiding the tumor rejection, we analyzed the percentages of CD4+ and CD8+ cells from the total spleens of mice from each group, and the percentages of these cells specifically from the lymphocyte region. No significant differences were found in the total percentages of CD4+ (black bars) or CD8+ (gray bars) cells between the three groups (Fig 24A). Likewise, gating on the lymphocyte region and analyzing the percentages of CD4+ and CD8+ T cells also provided no significant differences between the groups (Fig. 24B).

In an effort to devise an AIT treatment that could avoid the use of MDSC-depleting antibody in vivo, we next wanted to determine of the migration patterns of adoptively transferred T cells would have an impact on their effectiveness. Since we have previously observed very high numbers of MDSC in the spleens of tumor bearing mice, while observing very few at the tumor site (Fig. 5), we hypothesized that transferring CD62L-
Figure 24: Adoptive immunotherapy treatment and with or without Gr1+ depletions does not increase CD4+ or CD8+ T cells in the spleens of FVBN202 mice.

FVBN202 mice that received adoptive transfer of T cells expanded in alternating cytokines (AIT alone mice), received AIT along with depletion of Gr1+ cells (AIT+Gr1- mice), or received MMC and cyclophosphamide alone (MMC alone) were sacrificed and their spleen cells were stained for CD4 and CD8. (A) The percentage of the total splenocytes that is made up of CD4+ or CD8+ cells in each of the 3 treatment groups. (B) The percentage of CD4+ or CD8+ cells from within the lymphocyte region that was present in each of the 3 treatment groups. Data are averages of 2 mice per group +/- SD.
effector cells would allow them to go directly to the tumor site without first homing to the immunosuppressive environment of the secondary lymphoid organs. In order to test this, FVB mice were inoculated with MMC cells as before and were sacrificed 22 days later. Spleens were removed and cells were stimulated with B/I and expanded in alternating gamma chain cytokines as previously. FVBN202 mice were pre-treated 24 hours in advance with CYP as before. On day 7 of the donor cell expansion, FVBN202 recipient mice were divided into 3 groups, those receiving no treatment, those receiving the adoptive transfer of CD62L+ cells, and those receiving the adoptive transfer of CD62L- cells. All recipient mice were challenged with 3\times10^6 MMC. Donor T cells were then stained with a PE labeled anti-mouse CD62L antibody and were subjected to positive magnetic bead selection of PE-labeled cells. Both the positively selected PE-labeled cells (CD62L+ fraction) and the non-PE labeled cells (CD62L- fraction) were collected; 70\times10^6 CD62L+ or CD62L- cells were injected i.v. into mice receiving treatment. Figure 25A shows that the three groups of mice, those receiving no treatment (squares), those receiving CD62L+ T cells (triangles), and those receiving CD62L- T cells (diamonds) exhibited extremely similar rates of tumor growth. Serum was collected from mice on day 21 and tested for antibody responses against neu ECD. Figure 25B shows that, in the absence of any MDSC depletion, none of the groups showed an antibody response against neu, regardless of the type of T cell transfer.
Figure 25: Adoptive transfer of CD62L- T cells does not prevent T cell suppression by MDSC

(A) Tumor growth chart of FVB/N202 recipient mice that were pretreated with CYP as before and then received either no treatment (squares), adoptive transfer of CD62L+ T cells expanded in alternating cytokines (triangles) or CD62L- Y cells expanded in alternating cytokines (diamonds). (B) ELISA against the extracellular domain of HER2/neu using serum taken on day 21. Data are the averages of 4 mice per group, each done in duplicate.
A. Tumor growth in mice receiving CD62L+ or CD62L- AIT

![Graph showing tumor growth over weeks after inoculation.](image)

B. IgG1 response against HER2/neu ECD

![Graph showing IgG1 response at different dilutions.](image)

- ▲ Mice given CD62L+ T cells
- ■ Mice given CD62L- T cells
- □ Mice given no T cells
Gemcitabine is an Effective Inhibitor of MDSC in Tumor Bearing FVBN202 and Allows for the Complete Rejection of Neu Positive Mammary Tumors.

Since Gemcitabine has been reported to decrease MDSC in vivo without affecting lymphocyte populations (70), we hypothesized that combining Gemcitabine treatment with AIT may result in a more clinically relevant means of inhibiting this population in vivo, and would therefore result in the success of AIT treatments. T cells were therefore harvested from FVB donors and were stimulated and expanded in alternating gamma chain cytokines as previously. All FVBN202 recipient mice were treated 48 hour prior to AIT transfer with CYP (100mg/kg). Twenty-four hours prior to transfer, recipient FVBN202 mice were challenged with 3X10^6 MMC cells. On the day of adoptive transfer, FVBN202 mice were divided into four groups: mice receiving no treatment (CYP), mice receiving AIT only (AIT), mice receiving Gemcitabine only (Gem), and mice receiving AIT transfer along with Gemcitabine treatment (Gem+AIT). 70X10^6 expanded donor T cells were transferred i.v. into recipient mice receiving AIT. Gemcitabine treatment was administered i.p. at a dose of 75mg/kg per mouse on days 6, 11, and 16. To test the efficacy of Gemcitabine in diminishing MDSC in vivo in tumor bearing mice, blood was drawn at several time points and analyzed by flow cytometry for the presence of CD11b+Gr1+ cells. By day 8 after tumor inoculation, mice not receiving Gemcitabine had increased MDSC levels from 0.885% of the total blood on day 0 to 2.55% of the total blood on day 8 (Fig 26A). In contrast, mice receiving Gemcitabine, either alone or with AIT, showed more modest increases of MDSC on day 8. The Gemcitabine alone group increased total levels of MDSC from 0.39% on day 0 to 0.87% on day 8 and the Gemcitabine with AIT group
Figure 26: Gemcitabine inhibits the accumulation of MDSC in the blood of MMC-challenged FVBN202 mice

(A) Percentages of MDSC in the peripheral blood on days 0 and 8 as determined by flow cytometry staining for CD11b and Gr1. Data are averages of 2-4 mice per group +/- SD. (B) Percentages of MDSC in the peripheral blood on day 14 as determined by flow cytometry staining for CD11b and Gr1. Data are averages of 2-3 mice per group +/- SD.
increased levels of MDSC from 0.62% on day 0 to 1.23% on day 8, thus showing a stunted accumulation on day 8 in mice that had received one injection of Gemcitabine at that time (Fig 26A). By day 14, after 2 Gemcitabine injections, there was a significant decrease in MDSC in both groups of mice receiving Gemcitabine treatment (Fig 26B). Untreated mice had an average of 15.5% MDSC out of their total peripheral blood leukocytes, whereas mice treated with Gemcitabine alone had only 2.61% MDSC (P=0.026 compared to untreated) and mice given Gemcitabine with AIT had 0.33% MDSC (P=0.017 compared to untreated). Mice receiving both Gemcitabine and AIT also showed significantly reduced levels of MDSC when compared to mice receiving Gemcitabine alone (P=0.0004, Fig. 25B), presumably because of the decreased tumor burden that was becoming apparent by day 14 in mice treated with Gemcitabine and AIT (Fig 27A). Figure 27A shows that by day 18, mice receiving Gemcitabine injections and AIT exhibited significantly reduced tumor volumes (P=0.004 when compared to CYP control mice, P=0.009 when compared to AIT mice, and P=0.024 when compared to Gem mice). The differences in tumor volumes were also significantly different between the Gem+AIT group and all other groups on days 21 and 24. Additionally, by day 24, mice receiving Gemcitabine alone also exhibited a significant reduction in tumor volume as compared to mice receiving AIT alone (P=0.037). By day 28, all five mice treated with Gem+AIT were completely tumor free. In addition to tumor volumes, we thought it important to monitor weights, to evaluate if the treatment was well tolerated. Figure 27B shows that, by week 4, the weights of mice in the Gemcitabine alone group had dropped significantly compared to those in the Gem+AIT group (P=0.015). It is also noteworthy that during the fourth week, mice treated with
Figure 27: Gemcitabine combined with AIT causes complete tumor regression, along with increased weight and survival of FVBN202 mice

(A) FVBN202 mice were treated with Cyp (100mg/kg), challenged with MMC tumors 24 hours later, and administered 70X10^6 adoptively transferred T cells 24 hours after that.

Control mice were not given either AIT or Gemcitabine (Cyp, diamonds). Mice given Gemcitabine were given 1.5mg i.p. on days 6, 11, and 16. Mice given AIT alone (Gem) are indicated by triangles, mice given AIT alone by squares, and mice given both Gem and AIT (Gem+AIT) by circles. Tumor growth was measured with a digital caliper. Data represent averages of 3-5 mice per group +/- SEM. (B) Mice were treated as above and were weighed every week. Data are averages of 3-5 mice per group +/- SEM. (C) Mice were treated as above and survival curve was plotted according to the percentage of mice in each group that were still alive during each week of the experiment.
A. Tumor growth in mice treated with Gemcitabine, AIT, or both

B. Weights of mice treated with Gemcitabine, AIT, or both

C. Survival of mice treated with Gemcitabine, AIT, or both
Gemcitabine alone had significantly reduced weights as compared to mice receiving AIT as well (P=0.012). Weeks 5 and 6 showed a continued significant reduction in the weights of mice treated with Gemcitabine alone as compared to mice receiving Gem+AIT (P=0.022 in week 5 and 0.005 in week 6). The survival of all four groups of mice is shown is Figure 27C, with the CYP alone control group reaching 0% survival by the end of week 5, and the Gemcitabine alone and AIT alone groups reaching 0% survival by the end of week 6. It is noteworthy that although the use of Gemcitabine alone offered some reduction in tumor volume, the significant reductions in weight coupled with a lack of increased survival indicates that despite the role of Gemcitabine as an anti-tumor drug, this drug alone is not sufficient. However, the combination of Gemcitabine with AIT results in potent anti-tumor activity.

Since the depletion of MDSC causes a restoration of antibody responses against the ECD of neu in tolerant mice, we wanted to determine if MDSC inhibition by Gemcitabine had the same effect. Serum was taken from FVBN202 mice that received either no treatment (CYP), AIT alone (AIT), Gemcitabine alone (Gem), or Gemcitabine and AIT (Gem+AIT). Serum was tested for antibody responses against ECDII and ECDIV of the extracellular domain of neu. Optical density readings showed that only the group that received both Gemcitabine and AIT generated an antibody response against ECDII (Fig. 28A). Analysis of antibody responses from individual mice within the Gem+AIT group showed a large variation that correlated with the amount of time it took individual mice to fully reject MMC tumors. The mouse with the highest antibody response, mouse 1, (O.D. of 3.32 at a 1:20 dilution) took 27 days to fully reject the tumor, and therefore still had an
Figure 28: Only mice given AIT and treated with Gemcitabine have an antibody response against subdomain II of the ECD of HER2/neu

(A) Mice were treated with Cyp (MMC+Cyp) alone, Gemcitabine (Gem) alone, AIT alone (AIT), or Gemcitabine with AIT (Gem+AIT), as previously. Serum was collected on day 21 and ELISA's run against subdomain II of the ECD of HER2/neu. Data are averages of 2-4 mice per group, each sample run in duplicate +/- SEM with *** indicating that the Gem+AIT is significant as compared to each of the other 3 treatment groups. (B) Individual antibody responses against subdomain II of the ECD of HER2/neu from the 5 mice in the Gem+AIT group. Data are averages of duplicate wells +/- SEM. (C) Antibody responses against subdomain IV of the ECD of HER2/neu from each of the four treatment groups above. Data are averages of 2-4 mice per group, each mouse run in duplicate, +/- SEM.
active immune response at the time serum was taken. Mouse 3, which had an O.D. of 1.5 at a 1:20 dilution took 23 days to reject the tumor, whereas mouse 4, with an O.D. of 0.82, took 17 days to reject the tumor, and the 2 mice with the lowest antibody responses (mouse 2 and mouse 5) both took only 8 days to fully reject their MMC tumors. Therefore, the immune responses of some of these mice had fully contracted by the time that serum was taken, thus accounting for the large amount of variation in antibody responses (Fig. 28 A,B). Conversely, no substantial antibody response was seen towards ECDIV of the neu ECD (Fig 28 C). This correlates with a previous finding in the lab that after challenge with MMC tumors, FVB mice mount an antibody response consisting almost entirely of antibody against ECDII (Fig. 29). These mice successfully reject MMC tumors without responses against ECDIV. Therefore antibody responses against ECDII are protective against MMC tumors, while antibody responses against ECDIV are not needed.

Since 100% of the mice that were treated with both Gemcitabine and AIT fully rejected their MMC tumors, we wanted to determine if these mice could rejected a second challenge with MMC without adoptively transferred T cells. Mice were therefore challenged on the contralateral side with $3 \times 10^6$ MMC cells 74 days after the initial MMC challenge. Mice were treated on days 6 and 11 with 75mg/kg of Gemcitabine. As indicated in Figure 30, MMC tumors never reached palpable status and were not able to be measured. By day 16 all mice were completely tumor free. Serum was taken from all 5 mice 12 days after the re-challenge and tested for antibody responses against ECDII of neu. As shown in Figure 31A, mouse 1 and mouse 3 exhibited strong antibody responses against ECDII, whereas mouse 2 and mouse 4 had weak antibody responses and mouse 5
Figure 29: FVB mice that have successfully rejected MMC tumors display IgG1 antibody responses only against subdomain II of HER2/neu.

Serum from 10 FVB mice was taken following rejection of MMC challenge. ELISA plates were coated with 4μg/mL of sub-domain I, subdomain II, or subdomain IV of the extracellular domain of HER2/neu and ELISA performed for the detection of IgG1 antibody responses. Mean antibody titers were calculated.
Figure 30: Mice treated with Gemcitabine and AIT can reject further challenge with MMC

Five mice that previously were treated with Gemcitabine and AIT and completely rejected MMC tumors, were re-challenged 74 days after the original challenge on the contralateral side with $3 \times 10^6$ MMC (Day 0). Re-challenged mice were treated with 1.5 mg of Gemcitabine on days 6 and 11. Tumors did not grow in any of the five mice, therefore, 18 days after the first re-challenge, 3 of the five mice were re-challenged again the contra-lateral side with $3 \times 10^6$ MMC (Day 18) and were not treated further with Gemcitabine. Tumor growth was measured again starting 7 days after the second challenge. Black arrows indicate days of re-challenge with MMC.
Tumor growth of individual mice treated with AIT and Gemcitabine and subsequently re-challenged

Mouse number

- 1
- 2
- 3
- 4
- 5
had a negligible antibody response. Based on these data, the 2 mice with the lowest antibody responses (mouse 4 and mouse 5) were sacrificed 18 days after the MMC re-challenge. Splenocytes from these mice were cultured for 24 hours either alone (-) or in the presence of irradiated MMC cells (+) at an E:T ration of 10:1. Supernatants were collected and assayed for IFN-γ by ELISA. Figure 31B shows very low or undetectable levels of IFN-γ in wells containing media alone, MMC alone, or splenocytes alone that were taken from a naïve FVBN202 mouse or from either of the mice that had rejected MMC challenge (Re-challenged AIT). While the addition of MMC did not cause a significant increase in IFN-γ secretion by naïve splenocytes, a large increase in IFN-γ by splenocytes taken from mice that had rejected 2 MMC challenges was observed. The baseline level of IFN-γ secretion from the splenocytes of each mouse was subtracted from the level of IFN-γ secretion in the presence of MMC. After this normalization, it was determined that IFN-γ secretion by splenocytes from both re-challenged AIT mice in the presence of MMC was significantly higher (P<0.001) than that seen by splenocytes from the naïve control in the presence of MMC (Fig. 31B). To determine if the 3 remaining mice could reject an MMC challenge without Gemcitabine, these mice were challenged for a third time with 3X10⁶ MMC cells 18 days after the second challenge (challenges are indicated by the black arrows in Fig. 30). This was now day 0 of the third challenge. On day 7 of the third challenge, 2 mice (mouse 1 and mouse 3) were tumor free and one mouse (mouse 2) had a tumor size of 21.1mm³. By 14 days after the third challenge, this tumor had shrunk to 10.4mm³ and by day 21 was no longer visible (Fig. 30). To determine if these mice could mount an antibody response without the aid of Gemcitabine, serum was taken sixteen days
Figure 31: Mice receiving AIT and Gemcitabine treatment mount an immune response against subsequent MMC challenge

(A) Serum was taken 12 days after FVBN202 mice that had received AIT and Gemcitabine had rejected a second challenge with MMC (as described in Figure 29). ELISA was run against subdomain II of the HER2/neu ECD and read at 450nm. Data are averages of each sample run in duplicate +/- SD (B) Two of the 5 mice from above were killed and their splenocytes were cultured in duplicate either alone (-) or at a 10:1 E:T ratio with MMC cells (+). FVBN202 naive splenocytes were used as a control. Supernatants were collected after 24 hours and used in an ELISA for the detection of IFN-γ. Data are averages of duplicate samples +/- SD. Triple asterisks (***) indicate that after subtracting the baseline value of IFN-γ secretion by splenocytes alone, the resultant value achieved a p-value <0.001 as compared to MMC alone, media alone, and naive+MMC alone.
after the third MMC challenge. An ELISA against ECDII of neu showed that all three mice indeed had an antibody response (Fig. 32). It is interesting to note, however, that the mouse with the lowest antibody response (mouse 2 indicated by squares) is the same mouse that grew a small tumor after the third challenge (mouse 2 indicated by squares in Fig. 30). Therefore, AIT with Gemcitabine treatment not only causes full rejection of initial tumors, but also causes the rejection of subsequent MMC challenges, and creates long-lasting immunological memory capable of rejecting neu+ tumors even in the absence of Gemcitabine.

Long term follow-up of the 3 remaining mice treated with Gemcitabine and AIT revealed that by day 148 one mouse (mouse 3) had a tumor of 1076 mm$^3$. Isolation of the tumor and staining for neu expression showed this tumor to be of the ANV relapsed phenotype (Fig. 33, left panel vs. right panel) with a small amount of residual neu expression but dramatic downregulation from the highly neu+ MMC cells (Fig. 33 left vs. center).
Figure 32: FVBN202 mice challenged for a third time with MMC and receiving no further Gemcitabine treatment mount an antibody response against sub-domain II of neu.

Three of the FVBN202 mice that had rejected 2 MMC challenges with Gemcitabine treatment were challenged for a third time with 3x10^6 MMC (see Fig 29) and were given no further treatments. Sixteen days after the third challenge, serum was taken and used in an ELISA to detect IgG1 responses against sub-domain II of neu. Optical density was read at 450nm. Data are averages of each sample run in duplicate +/- SD.
Antibody response against subdomain II of ECD sixteen days after second re-challenge

Mouse number
1
2
3
Figure 33: Tumor relapse in mouse that received Gemcitabine and AIT and rejected 3 challenges of neu+ MMC cells is of the ANV phenotype.

Mouse 3 had a relapsed tumor of 1076mm² 148 days after the first MMC inoculation. The tumor was harvested and homogenized and cultured for 48 hours in complete medium to allow non-tumor cells to die. Cells were then detached (EDTA with 0.5% trypsin), stained for surface expression of rat neu, and analyzed by flow cytometry (left; dotted line indicates background from the secondary antibody alone). Neu expression is compared to that normally seen on MMC cells (center) and ANV cells (right).
III. Conclusions and Significance

These studies have found that the adoptive transfer of B/I activated and IL-2 expanded T cells results in statistically significant tumor inhibition and increase in antibody response in FVBN202 mice only when their Gr1+ cells are depleted in vivo. Importantly, the depletion of MDSC was not effective in the absence of transferred T cells showed no benefit, showing that tumor rejection requires both the presence of highly effective anti-tumor T cells and the absence of MDSC. Furthermore, a correlation exists between the efficacy of the Gr1+ depletions and the observed anti-tumor response. Efforts to generate T cells with greater cytotoxicity towards MMC were successful through the use of the gamma chain cytokines IL-7 and IL-15 with a one time pulse of IL-2. We conclude that this is a superior method of T cell expansion based on increased viability, cytotoxicity, and expansion levels as compared to expansion in IL-2 alone. The pivotal role of MDSC in inhibiting the function of adoptively transferred T cells was confirmed after transfer of T cells expanded in alternating gamma chain cytokines resulted in tumor inhibition and anti-neu antibody responses only when these cells were depleted, as previously. Efforts to evade the large population of MDSC in the spleen through the transfer of CD62L- T cells were unsuccessful and leads us to conclude that adoptively transferred T cells, regardless of their expansion method, in vitro cytotoxicity, or migratory patterns, cannot mediate effective tumor regression in vivo in the presence of MDSC and that the elimination of these cells in vivo results in effective tumor rejection or inhibition in an otherwise tolerant mouse model.
The use of Gemcitabine as a clinically relevant means of inhibiting MDSC in vivo in conjunction with AIT was found to be superior to treatment with either AIT or Gemcitabine alone and was far superior to AIT in conjunction with depleting antibodies. 100% of mice receiving AIT with Gemcitabine exhibited complete rejection of the primary tumor challenge. Of great significance is the fact that two subsequent tumor challenges were also rejected by these mice, showing long-lasting immunological memory, which was confirmed by the presence of high antibody titers against neu and strong IFN-γ responses against MMC.

These studies have therefore identified MDSC as the major mediator of suppression against adoptively transferred T cells and have developed a clinically achievable means of achieving tumor rejection through the use of Gemcitabine as an MDSC inhibitor. These findings are of great significance given the fact that adoptive immunotherapy has exhibited extremely limited results for primary breast cancers (113), with some difficulty encountered in expanding populations of cells with high percentages of CD8+ T cells and cytotoxic activity (144). While there has been some success in the treatment of metastatic lesions of breast cancer using AIT (230), overall clinical response rates and long-term survival rates have been extremely low (231). We therefore propose that AIT in human breast cancer patients, who have been observed to have increased levels of circulating MDSC, be combined with the use of Gemcitabine for increased tumor rejection and long-lasting immunity.
Chapter 2

I. Study Rationale

MDSC have been reported to suppress T cell responses by a wide variety of mechanisms, which include soluble factors such as nitric oxide or IDO (187, 232), but some have reported contact-dependency between T cells and MDSC (184, 233). Given the important role that MDSC play in suppressing AIT, we sought to determine the mechanism of suppression by MDSC in our model and to characterize the existence of possible effector molecules expressed either on the surface of, or within these cells. Suppression by specific subsets of MDSC was also investigated to further pinpoint the cells responsible for immune suppression so they may more precisely targeted in a therapeutic setting. Given the equal tumor growth seen in mice treated with AIT consisting of CD62L+ and CD62L- cells, we hypothesized that MDSC may be exhibiting wide-spread suppression through secreted soluble factors instead of relying on contact-dependent mechanisms, since CD62L- T cells would not have come into direct contact with as many MDSC as CD62L+ T cells. Therefore MDSC were isolated from tumor-bearing animals, RNA extracted and reverse transcribed, and tested for the expression of various suppressive soluble molecules. These data indicated that the tested soluble factors are most likely not involved in suppression my MDSC in our model, which lead us to investigate possible roles of several
inhibitory molecules in mediating what we found to be contact-dependent suppression by MDSC.

II. Results

**MDSC in Tumor-Bearing FVBN202 Mice Suppress anti-CD3/CD28 Mediated Proliferation of both CD4+ and CD8+ T cells**

To determine the mechanisms by which MDSC suppress anti-tumor immune responses, splenocytes were isolated from MMC tumor-bearing and tumor-free FVBN202 mice. Tumor-bearing mice had a large influx of MDSC in their spleens as compared to tumor-free mice (Fig. 5). Splenocytes were labeled with the thymidine analog BrdU and stimulated with antibodies against CD3 (10μg/mL, plate-bound) and CD28 (1μg/mL, soluble), or were left unstimulated to serve as a control. After 3 days, cells were stained using anti-CD4 and anti-CD8 antibodies and analyzed for BrdU uptake by flow cytometry. We found significantly higher proliferation of CD4+ T cells from tumor-free mice compared to those from tumor-bearing animals (Fig. 34A left panel, 91% vs. 59%, P=0.002). The same trend was seen for CD8+ T cells (Fig. 34A right panel, 93% vs. 70%, P=0.001). Similar trends were detected when analyzing absolute numbers of CD4+ and CD8+ T cells (Fig. 34B left panel). Figure 34B shows that the average number of BrdU+CD4+ and BrdU+CD8+ T cells from tumor-free mice is 3.3-fold higher than that of BrdU+CD4+ and BrdU+CD8+ T cells from tumor-bearing mice (P=0.003 for CD4+ and P=0.0004 for CD8+ T cells). To confirm that the suppression of T cell proliferation in FVBN202 splenocytes was caused by the presence of elevated MDSC, we depleted MDSC
Figure 34: MDSC's suppress CD3/CD28 induced proliferation of T cells from FVBN202 mice

(A) Splenocytes from tumor bearing FVBN202 mice, tumor free FVBN202 mice, or tumor bearing FVBN202 mice depleted of Gr1+ cells in vitro were pulsed with BrdU at the manufacturer's recommended concentration and 2x10^5 cells were cultured in 96 well plates for 3 days with 10ug/mL of plate-bound anti-CD3 and 1ug/mL of soluble anti-CD28. After 3 days, cells were harvested and stained for CD4 and CD8, and then stained intracellularly for BrdU incorporation according to the manufacturer's protocol. Bar graphs represent the percentage of gated CD4+ T cells that incorporated BrdU (left column) and the percentage of CD8+ gated T cells that incorporated BrdU (right column). (B) Analysis of the absolute number of CD4+ (left column) and CD8+ (right column) T cells that incorporated BrdU after 3 days of anti-CD3 and CD28 stimulation as above.
*in vitro* from the splenocytes of tumor-bearing animals. As seen in Figure 34A, the depletion of MDSC (Tumor Bearing-MDSC) significantly restored the proliferative responses of both CD4+ (87% BrdU+) and CD8+ (92% BrdU+) T cells over those seen in total splenocytes from tumor-bearing mice ($P=0.028$ for CD4+ and $P=0.009$ for CD8+ T cells). Similar trends were found while comparing the absolute numbers of CD4+ and CD8+ T cells, showing a 3.1-fold increase in proliferation in the CD4+ population ($P=0.027$) and 2.5-fold increase in the CD8+ population ($P=0.012$) over total splenocytes from tumor-bearing mice (Fig. 34B).

**CD11b+Ly6G-Ly6C+ MDSC are Suppressive, while CD11b+Ly6G+Ly6C+ Cells are not**

It has recently been reported that various subsets of MDSC exist and can be distinguished based on their differential expression of two epitopes of the Gr1 molecule; Ly6G, corresponding to a granulocytic/neutrophil phenotype, and Ly6C, corresponding to a monocytic phenotype (171, 215, 216). We indeed found two different subsets of MDSC in MMC tumor-bearing FVBN202 mice. In order to determine if these subsets have different functions in our model, splenocytes were isolated from FVBN202 mice bearing neu+ MMC tumors and were stained for CD11b, Ly6G, and Ly6C. CD11b+ cells were sorted into Ly6G+Ly6C+ and Ly6G-Ly6C+ populations. Sorted MDSC were then added at a 1:2 ratio to FVB naïve splenocytes with anti-CD3/CD28 stimulation for 3 days. As shown in Figure 35A-B Ly6G-Ly6C+ subsets inhibited T cell proliferation whereas Ly6G+Ly6C+ subsets did not display any suppressive activity. Representative flow cytometry staining for BrdU incorporation into CD4+ T cells showed 57.5% BrdU+ cells in culture with
Figure 35: Only MDSC that are Ly6G−Ly6C+ are suppressive against anti-CD3/CD28-induced proliferation

(A) Splenocytes from tumor-bearing FVBN202 mice were triple stained for the surface markers CD11b, Ly6G, and Ly6C and were subjected to fluorescence activated cell sorting. Cells were sorted into two populations: CD11b+Ly6G+Ly6C+ and CD11b+Ly6G−Ly6C+. Sorted cells were then co-cultured at a 1:2 ratio with FVB naïve splenocytes stimulated in 96 well plates with anti-CD3 (plate-bound, 10 μg/mL) and anti-CD28 (soluble, 1 μg/mL). Control well contained splenocytes alone with no MDSC. All wells were pulsed with BrdU at the beginning of the culture period. Representative plots show the percentage of CD4+ cells on day 3 that also stained positive for intracellular BrdU incorporation. Bar graph is representative of 2 separate experiments, and shows the average percent BrdU incorporation into CD4+ cells as compared to control wells normalized to 100%. (B) BrdU incorporation into CD8+ cells on day 3 as described.
A. Proliferation in CD4+ cells

B. Proliferation in CD8+ cells

Inhibition of CD4+ T cells by MDSC subsets

Inhibition of CD8+ T cells by MDSC subsets
medium alone, 55.4% when in the presence of sorted CD11b+ Ly6G+Ly6C+ cells, and only 13.0% proliferation when cultured with CD11b+Ly6G-Ly6C+ cells (Fig. 35A). Repeated experiments confirmed these results, with the Ly6G-Ly6C+ fraction causing a 70% inhibition of CD4+ T cell proliferation when compared to the medium alone control, in which proliferation was normalized to 100% (100% BrdU+ in media alone vs. 30.1% BrdU+ in cultures containing Ly6G-Ly6C+ cells, \( P=0.008 \)). In contrast, there was no significant inhibition in cultures containing Ly6G+Ly6C+ cells (96.9% BrdU+, Fig. 35A). Similar results were found in CD8+ T cells, with representative BrdU staining showing 38.1% and 51.6% proliferation in splenocytes cultured with medium alone or with Ly6G+Ly6C+ cells, respectively (Fig. 35B). Strikingly, splenocytes cultured with sorted Ly6G-Ly6C+ cells showed only 12.2% BrdU+ CD8+ T cells. Normalizing the proliferation in control splenocytes alone to 100% shows a 62% inhibition of proliferation in CD8+ T cells cultured with Ly6G-Ly6C+ cells from splenocytes alone (\( P=0.008 \)), whereas no significant inhibition of proliferation was seen in CD8+ T cells cultured with Ly6G+Ly6C+ cells (Fig. 35B).

**Suppressive MDSC Require Direct Cell-to-Cell Contact that is Independent of LFA-1/ICAM and PD-1/PD-L1 interactions to Inhibit the Proliferation of CD4+ and CD8+ T cells**

To further characterize the suppressive function of MDSC, we sought to determine if the anti-proliferative effects of MDSC on T cells may be mediated by the release of immunosuppressive soluble factors or by contact-dependent mechanisms. MDSC were
isolated from tumor-bearing mice by using a magnetic bead selection kit to positively select for PE-labeled Gr1+ cells. RNA was extracted and reverse transcribed. Resultant cDNA was analyzed via PCR for the expression of the immunomodulating cytokine IL-10 (234, 235). Expression of mRNA for IL-10 was visible in both samples, but was relatively faint (Fig 36). We also wanted to determine if there may be differential expression of immunosuppressive molecules between the two subsets of MDSC. RNA was therefore extracted from sorted populations of CD11b+Ly6G+Ly6C+ and CD11b+Ly6G-Ly6C+ cells from the spleens of tumor-bearing mice and reverse transcribed. The expression of indoleamine 2,3-dioxygenase (IDO), an immunosuppressive enzyme that can inhibit T cell responses and promote immune tolerance through the breakdown of tryptophan was assessed by PCR (186, 187). After normalizing the data to the actin control, we found that both the Ly6G+Ly6C+ fraction and the Ly6G-Ly6C+ fraction displayed extremely low levels of IDO (Fig 37). In contrast, both fractions expressed TGF-β to a similar level after normalization, which may be secreted or surface-bound and is an important modulator of T cell tolerance, with mice lacking TGF-β exhibiting T cell hyper activation, even in the absence of foreign antigen (236-239) (Fig 37). Since expression of IDO was almost negligible and since TGF-β expression was observed to be similar in both populations, it is unlikely that either of these molecules is the primary means of T cell suppression by MDSC. We therefore wondered if suppression by MDSC may be achieved through direct cell-to-cell contact. Therefore, CD11b+ cells were depleted from the splenocytes of tumor-bearing and tumor free mice and equal numbers of lymphocytes (5X10^5) were stimulated in the lower chamber of a plate containing a Transwell insert. CD11b+ cells
Figure 36: MDSC express low levels of IL-10

Gr1+ cells were magnetically selected from the splenocytes of FVBN202 mice bearing MMC tumors. RNA was extracted from Gr1+ cells and was reverse transcribed into cDNA. PCR was performed for IL-10 and the control gene GAPDH. Representative samples from 2 individual mice are shown.
FVB/N202 mice bearing MMC tumors

GAPDH

IL-10
Figure 37: Expression of IDO and TGF-β in MDSC subsets

Splenocytes from tumor bearing FVBN202 mice were harvested and triple stained with antibodies against the surface antigens CD11b, Ly6G, and Ly6C. Cells were sorted into two populations of CD11b+ cells: Ly6G+Ly6C+ and Ly6G-Ly6C+. RNA was extracted from each population and reverse transcribed. PCR analysis was done for the expression of IDO, TGF-β, and actin as an internal control.
enriched from tumor-bearing mice were then added to the lower chamber of the plate, or were added to the top chamber of the Transwell insert, where they were separated from the lymphocytes by 8.0µm pores. Staining for BrdU incorporation on day 3 showed potent inhibition of both CD4+ and CD8+ T cells when CD11b+ cells were added back to the cultures, but only in the absence of a Transwell insert (Fig. 38A). Gray bars indicate wells to which CD11b+ cells from tumor-bearing mice were added, whereas black bars are indicative of the proliferation seen in cultures of CD11b-depleted splenocytes alone. When CD11b+ cells were added directly to the splenocytes (no Transwell), the proliferation of CD4+ cells decreased from 80.5% to 50% (Fig 38A, P=0.001). On the other hand, the addition of CD11b+ cells to the top of a Transwell insert had no effect on proliferation of CD4+ cells (90.5% BrdU+ in splenocytes alone vs. 90% BrdU+ with CD11b+ cells). A similar trend was seen in CD8+ cells, with the addition of CD11b+ cells directly to the culture causing a reduction in BrdU+ staining from 88% to 53% (P=0.01, Fig. 38A). In contrast, the addition of CD11b+ cells to the top of a Transwell insert resulted in no reduction of proliferation, with CD8+ splenocytes alone staining 94.5% BrdU+ and CD8+ splenocytes staining 89% BrdU+ (Fig 38A). Analysis of the absolute cell numbers recapitulated the above results; with the proliferation of CD4+ cells reduced significantly only when CD11b+ cells were added directly to the culture (Fig 38B).

Since it has been reported that MDSC from tumor free animals are not suppressive (167), we decided characterized MDSC in tumor free and tumor bearing animals to determine if there were important differences that may account for the suppressive capability in tumor-bearing mice. We looked at the expression of Annexin V, H-2^q, Qa1,
Figure 38: MDSC's suppress T cell proliferation in a contact-dependent manner

Splenocytes from FVBN202 mice were depleted of CD11b+ cells in vitro prior to culture. 2x10^5 cells were then plated on the bottom portion of wells containing a transwell insert, or not, with 10μg/mL plate bound anti-CD3 and 1μg/mL soluble anti-CD28 and were pulsed with BrdU at the manufacturer's recommended concentration. MDSC isolated from a tumor bearing FVBN202 mouse (1x10^5) were then added to the top chamber of the transwell insert, or directly to the splenocyte. Cells were cultured for 3 days and were then stained for CD4, CD8, and BrdU incorporation, as before. (A) The percentage of gated CD4+ and CD8+ cells that incorporated BrdU, and (B) The absolute numbers of CD4+ and CD8+ T cells that incorporated BrdU during the 3 day stimulation were analyzed. Data are the mean of duplicate wells.
A.

BrdU+ CD4+ T cells

BrdU+ CD8+ T cells

*P = 0.001

Perc. (2x50)

no transwell transwell

no transwell transwell

P = 0.01

B.

BrdU+ CD4+ T cells

BrdU+ CD8+ T cells

*P = 0.001

Cell number x 10^6

no transwell transwell

P = 0.087
Figure 39: CD86 expression on MDSC in MMC tumor-bearing FVBN202 mice is significantly lower than that seen in tumor free FVBN202 mice.

Splenocytes from FVBN202 mice that were either tumor free (TF) or had an MMC tumor (TB) were homogenized and triple stained with antibodies against the surface antigens CD11b, Gr1, and CD86 and analyzed by flow cytometry. (A) Representative data of CD86 expression gated on CD11b+Gr1+ cells from the granulocyte region. Gray histogram indicates CD86 expression whereas the dotted line indicates the levels of background (autofluorescence) present in the sample. Data are representative of 3 separate samples. (B) Bar graph depicts the averages percent of gated MDSC that express CD86 from 3 mice in each group +/- SEM with background autofluorescence subtracted.
A. CD11b+Gr1+ granulocytes from the spleen

- Tumor free: 57.1%
- Tumor bearing: 35.8%

B. CD86 expression on MDSC

- TF: ~50%
- TB: ~20%

*p = 0.0499
CD11c, CD86 and CCR7 on MDSC from tumor bearing and tumor free mice. We found no significant differences in the expression of any of these molecules with the exception of CD86, which was found to be significantly decreased on MDSC from tumor-bearing mice (Fig 39).

We then hypothesized that, since T cells highly express the integrin LFA-1 (Fig 40A), and since the LFA-1/ICAM interaction between T cells and DC’s is necessary for T cell priming (240) expression of LFA-1 on MDSC could allow MDSC to interact with DC’s via ICAM-1 and cause DC’s to suppress T cells as well. Using flow cytometry, we found that 100% of CD11b+Gr1+ MDSC express LFA-1 (Fig. 40B). However, we also found LFA-1 to be highly expressed on CD11b-Gr1- cells of the granulocyte region as well (Fig. 40B). Therefore, interaction through the LFA-1/ICAM-1 axis is unlikely given the widespread expression of LFA-1 on non-MDSC as well as MDSC.

Programmed cell death 1 (PD-1), a member of the extended CD28/CTLA4 family, is an immunoinhibitory receptor expressed on activated T cells (241). PD-1 had two potential binding partners, PD-1 ligand 1 and PD-1 ligand 2 (PD-L1 and PD-L2, respectively). Both ligands can be expressed on activated dendritic cells and macrophages, and ligation of PD-1 by PD-L’s has been shown to inhibit TCR-induced proliferation, induce T cell anergy, or cause apoptosis (241-243). Flow cytometry of splenocytes from FVB mice that had rejected MMC cells showed low levels of PD-1 on the surface of CD8+ T cells (Fig 41). However, when these splenocytes were restimulated with MMC cells in vitro, PD-1 expression increased to 3.94% on CD8+ T cells, a 4.4-fold increase (Fig 41).
Figure 40: T cells and MDSC highly express LFA-1
Spleens from FVB/N202 tumor-bearing mice were homogenized and stained with antibodies against the surface antigens CD4, CD8, and LFA-1 (A) or CD11b, Gr1, LFA-1 (B) and analyzed by flow cytometry. Data are gated plots and are representative of 3-4 mice per group.
Figure 41: PD-1 is upregulated on CD8+ T cells after re-activation \textit{in vitro} by MMC cells

FVB mice were challenged with MMC and were sacrificed immediately after rejection of MMC. Splenocytes were then cultured in the presence or absence of irradiated MMC for 24 hours and were stained with antibodies against the surface antigens CD8 and PD-1 and analyzed by flow cytometry. Analysis is gated on CD8+ lymphocytes and is representative of 4 mice.
We therefore hypothesized that MDSC may express PD-L1 and that the PD-1/PD-L1 interaction may be the contact-dependent mechanism of MDSC suppression. MDSC were then tested for the expression of PD-L1. As shown in Fig. 42, neither CD11b+Gr1+ nor CD11b-Gr1- cells from the blood of FVBN202 mice showed any expression of PD-L1 or PD-1 (Fig 42), suggesting that PD-1/PD-L1 interaction is not the mechanism by which MDSC suppress activated T cells.
Figure 42: MDSC's from FVBN202 mice do not express PD-1 or PD-L1

(A) Peripheral blood cells from FVBN202 naïve mice were stained for CD11b, Gr1, and PD-1 or PD Ligand-1. Histograms are gated on CD11b+Gr1+ cells from the granulocyte region. Peripheral blood from FVBN202 tumor bearing mice was also stained and analyzed in the same way as above (data not shown) and results were similar. All data is representative of 4-5 mice.
III. Conclusions and Significance

From these studies we can conclude that MDSC require contact with T cells in order to suppress CD3/CD28-induced proliferation. Furthermore, we have identified a specific subset of CD11b+Ly6G-Ly6C+ cells that are responsible for mediating all observed suppression. This finding is of significance given the fact that it will enable the more direct targeting of MDSC in a therapeutic setting, while leaving the population of non-suppressive CD11b+Ly6G+Ly6C+ cells intact, therefore limiting toxic side effects. Indeed, we have seen some toxicity associated with the depletion of Gr1+ cells using monoclonal antibodies in vivo, probably because most immature progenitor cells in the bone marrow express the Gr1 molecule and complete depletion therefore reduces the potential production of mature neutrophils and myeloid-dendritic cells. The identification of a more specific subset of suppressive MDSC is therefore immensely important.

Although Gemcitabine does not selectively inhibit certain subpopulations of MDSC and shows little toxicity, resistance to chemotherapies is a widespread problem. Alternative methods of inhibiting specific subpopulations of MDSC should therefore be investigated for administration to Gemcitabine-resistant individuals. More extensive phenotyping of the CD11b+Ly6G-Ly6C+ population should be done to identify potential surface markers that may be unique to this population and therefore targeted with a humanized antibody. We further conclude that suppression of T cell proliferation by these MDSC in independent of IL-10, IDO, and TGF-β. Contact between MDSC and T cells is also independent of LFA-1/ICAM-1 interactions and PD-1/PD-L1 interactions. Other potential sites of contact between MDSC and T cells should be investigated.
Chapter 3

I. Study Rationale

We have observed large increases in MDSC in tumor-bearing mice, and therefore hypothesized that MMC-derived soluble factors were responsible for the increase in MDSC seen in vivo. We aimed to identify the particular cytokines or chemokines secreted by MMC that may contribute to this increase. To more specifically elucidate how MMC-derived cytokines or chemokines mediate the observed increase in MDSC, bone marrow cells were sorted into two pure populations: existing CD11b+Gr1+ MDSC and progenitor CD11b-Gr1- cells. Culture of these sorted populations as opposed to culture of total bone marrow cells would allow us to determine the effects of MMC-derived cytokines or chemokines both on the maintenance and proliferation of existing MDSC and on the generation of new MDSC from progenitor cells to determine if the observed increase in MDSC was a result of prolonged survival and/or proliferation of MDSC or to increased generation of MDSC de novo. Considering our previous finding that only one MDSC subset showed suppressive activity, we sought to establish the influence of various MMC-derived soluble factors on determining the proportions of these subsets. We hypothesized that the presence of MMC-derived soluble factors may specifically increase the proportion of suppressive MDSC. Lastly, we hypothesized that exposure to MMC-derived soluble factors may result in an inhibition of MHC class I and MHC class II molecules, as well as
various maturation markers, thus contributing to the inability of these granulocytes to fully mature and function properly as antigen presenting cells.

II. Results

**MMC-Derived Soluble Factors Cause the generation of MDSC**

To determine if soluble factors released by MMC cells are responsible for the increase in MDSC, MMC cells (10X10^6 cells/10mL) were cultured for 24 hours in RPMI1640 supplemented with 10% FBS. Supernatant was then removed and concentrated to a volume of 600µL. FVB mice (n=3) were then injected i.d. with 200µL of the concentrated supernatant. FVB control mice (n=3) were injected i.d. with 200µL of control medium alone. Mice were injected on three consecutive days, sacrificed on day 4 and their blood, spleens, and bone marrow were subjected to flow cytometry analysis. Figure 43A shows representative flow cytometry plots illustrating an increase in the overall granulocyte region of the spleen from 7% in RPMI-treated animals to 13% in conditioned medium (CM)-treated animals. Representative fluorescent staining for CD11b and Gr1 is depicted in Fig. 43B, with control animals having 17% CD11b+Gr1+ MDSC, while CM-treated animals had 55% MDSC in the granulocyte regions of their spleens. The difference in MDSC in the granulocyte region of the spleen from 7% in RPMI-treated animals to 13% in conditioned medium (CM)-treated animals. Representative fluorescent staining for CD11b and Gr1 is depicted in Fig. 43B, with control animals having 17% CD11b+Gr1+ MDSC, while CM-treated animals had 55% MDSC in the granulocyte regions of their spleens. The difference in MDSC in the granulocyte region of the spleen is statistically significant (P=0.003), as seen in Fig. 43C, with RPMI-treated mice averaging 21% MDSC and CM-treated mice averaging 54.7% MDSC. Differences in the percentage of MDSC in the total splenocytes were also significant, with MDSC comprising only 1.5% of the total spleens of RPMI-treated mice, while these cells comprised 7.33% of the total spleens of CM-treated mice.
Figure 43: MMC-derived soluble factors cause an increase in MDSC’s in the spleens of FVB mice

(A) Representative forward scatter vs side scatter flow cytometry analysis of the spleens of FVB mice that received intradermal injections on 3 consecutive days of either RPMI (containing 5% FBS) or with RPMI that had been conditioned with 10X10^6 MMC’s (in 10mL) for 24 hours. Media was removed and concentrated by centrifugation with a VivaSpin tube with a 10,000 kDa cutoff to a volume of 600uL. MMC conditioned medium (CM) was used to inject 3 FVB mice (200uL per mouse). Percentages reflect the percent of the whole spleen that is made up of granulocytes. (B) Representative flow cytometry plots of CD11b+Gr1+ cells from within the granulocyte region of the spleens of FVB mice that were treated as described above with either CM or RPMI. (C) Average percentages of CD11b+Gr1+ cells from the granulocyte regions of the spleens of FVB mice injected intradermally with RPMI or CM as described above. Data are representative of 3 mice per group. (D) Average percentages of CD11b+Gr1+ cells from the total spleens of FVB mice injected intradermally with RPMI or CM. Data are representative of 3 mice per group. (E) Averaged absolute number of CD11b+Gr1+ cells from the whole spleens of mice receiving RPMI or CM injections. Data are representative of 3 mice per group.
Likewise, calculation of the absolute number of MDSC in the spleen of each animal confirmed a significant increase in MDSC in CM-treated mice, with RPMI-treated mice having $1.8 \times 10^6$ total MDSC, while CM-treated mice had $8.6 \times 10^6$ total MDSC ($P=0.002$, Fig. 43D). We also wanted to determine if MDSC were increased in other compartments of the body in CM-treated mice. Therefore, blood was also taken and stained for CD11b and Gr1. Figure 44A shows an increase in the overall granulocyte region from 10% in RPMI-treated mice to 15% in CM-treated mice. Furthermore, representative data from within this granulocyte region shows an increase from 24% MDSC in RPMI-treated mice to 93% MDSC in CM-treated mice (Fig. 44B). Quantification of the above data shows a statistically significant difference between the average percentage of MDSC in the granulocyte region of the blood of RPMI-treated mice (21.5%) and CM-treated mice (78%, Fig 44C, $P=0.00025$). The percentage of the overall blood comprised of MDSC also showed a significant difference between RPMI-treated mice (1.4%) and CM-treated mice (10.1%, Fig. 44C, $P=0.0033$). Lastly, there was a substantial increase in the overall percentage of granulocytes in the bone marrow as well, with the bone marrow of RPMI-treated mice having 39% granulocytes and the bone marrow of CM-treated mice having 54% granulocytes (Fig. 45A). The composition of the granulocyte region was also significantly affected by treatment with CM, as control mice had 35% MDSC while CM-treated mice had 48% MDSC ($P=0.028$, Fig 45B,D). These results were also reflected in the overall percentage of MDSC in the bone marrow (29.3% in RPMI-treated mice vs. 38.7% in CM-treated mice, $P=0.0186$, Fig. 45C), as well as the
Figure 44: MMC-derived soluble factors cause an increase in MDSC in the blood of FVB mice.

(A) Representative forward scatter vs side scatter flow cytometer analysis of peripheral blood of FVB mice that received intradermal injections on 3 consecutive days of either RPMI (containing 5% FBS) or with RPMI that had been conditioned with 10×10⁶ MMC's (in 10mL) for 24 hours. Media was removed and concentrated by centrifugation with a VivaSpin tube with a 10,000 kDa cutoff to a volume of 600μL. MMC conditioned medium (CM) was used to inject 3 FVB mice (200μL per mouse). Percentages reflect the percent of the whole spleen that is made up of granulocytes. (B) Representative flow cytometry plots of CD11b+Gr1+ cells from within the granulocyte region of the blood of FVB mice that were treated as described above with either CM or RPMI. (C) Average percentages of CD11b+Gr1+ cells from the granulocyte regions of the blood of FVB mice injected intradermally with RPMI or CM as described above. Data are representative of 3 mice per group. (D) Average percentages of CD11b+Gr1+ cells from the total blood of FVB mice injected intradermally with RPMI or CM. Data are representative of 3 mice per group.
Figure 45: MMC-derived soluble factors cause an increase in MDSC in the bone marrow of FVB mice

(A) Representative flow cytometry plots of forward scatter vs side scatter of total bone marrow cells from FVB mice injected intradermally with RPMI containing 5% FBS or with RPMI conditioned with MMC supernatant as before. Gated regions and percentages reflect the percent of the total bone marrow cells that fall within the granulocyte region. (B) Representative flow cytometry plots of CD11b+Gr1+ cells from the total bone marrow population from FVB mice treated with RPMI containing 5% FBS or with MMC-conditioned medium as before. (C) The percentage of CD11b+Gr1+ cells in the total bone marrow population in significantly increased in mice treated with MMC-conditioned medium as compared to mice treated with control medium. (D) The percentage of CD11b+Gr1+ cells in the granulocyte region increases in the bone marrow of mice treated with MMC-conditioned medium over mice treated with control medium. (E) The number of CD11b+Gr1+ cells in bone marrow isolated from the tibias and femurs increases in mice that have received injections of conditioned medium over the values seen in mice treated with RPMI.
absolute number of MDSC recovered from the tibias and femurs (7X10^6 MDSC in RPMI-treated mice vs. 13X10^6 MDSC in CM-treated mice, P=0.0156, Fig. 45E).

To determine if injection with MMC-conditioned medium influenced MDSC in any other way, flow cytometry staining was done for the expression of the MHC class II molecule I-A^q and the MHC class I molecule H-2^q. We found that mice that were treated with CM had significantly lower surface expression of I-A^q than mice treated with RPMI (P=0.026, Fig. 46). We therefore wondered if MMC-derived soluble factors may be affecting antigen presentation on mature antigen presenting cells as well. Figure 47 shows the expression of I-A^q on CD11b+Gr1^- cells, revealing that MMC-conditioned media does, in fact, significantly reduce the expression of I-A^q on the surface of APC in mice treated with CM (76.6%) as compared to mice treated with RPMI (93.9%, P=0.0054).

To determine the most prevalent soluble factors secreted by MMC that may be contributing to the observed effects in vivo, a multiplex array detecting IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IFN-γ, TNF-α, GM-CSF, MCP-1, and VEGF was performed on MMC-conditioned media after a 24 hour culture. We found that high levels of GM-CSF, VEGF, IL-6 and MCP-1 were secreted by MMC (Fig. 48). Other cytokines were below detectable levels.

Since GM-CSF, VEGF, IL-6 and MCP-1 were identified to be secreted by MMC cells, we wanted to determine if administration any of the above cytokines could cause an increase in MDSC in naïve FVB mice. We therefore injected varying mice once daily with a dose of either 500ng of GM-CSF, 2500ng of GM-CSF, or 500ng of GM-CSF with 120U
Figure 46: Intradermal injection of MMC-conditioned medium causes a significant reduction in the expression of I-Aδ on the surface of CD11b+Gr1+ MDSC in the spleens of FVB mice.

FVB mice were treated as previously described with either MMC-conditioned medium (CM) or with RPMI containing 5% FBS. Spleens were harvested and cells stained for the simultaneous expression of CD11b, Gr1, and I-Aδ. (A) Representative data from 3 separate mice showing the expression of I-Aδ on CD11b+Gr1+ cells from the granulocyte region. (B) Quantification of the percentage of MDSC from the granulocyte region that express I-Aδ. Data are averages of 3 mice per group +/- SEM with background autofluorescence subtracted.
A. 
CD11b+Gr1+ granulocytes from the spleen

<table>
<thead>
<tr>
<th>RPMI</th>
<th>CM</th>
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<tr>
<td>51.9%</td>
<td>44.7%</td>
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B. 
Percentage of F4/80 positive MDSC from the granulocyte regions of FVB mice treated with MMC-conditioned medium or control medium

![Graph showing percentage of F4/80 positive MDSC](image-url)
Figure 47: Intradermal injection of MMC-conditioned medium causes a downregulation of the MHC class II related molecule I-A\(^d\) on CD11b+Gr1-DC's in the spleens of FVB mice.

FVB mice were treated as before with MMC-conditioned medium (CM) or with RPMI containing 5% FBS as a control. After three injections, spleens were harvested and stained for the surface markers CD11b, Gr1, and I-A\(^d\). (A) Representative data showing I-A\(^d\) expression of CD11b+Gr1- cells from the granulocyte region. Data are representative of 3 mice. (B) Quantification of I-A\(^d\) expression on CD11b+Gr1- cells from the granulocyte region. Data are the averages of 3 mice per group +/- SEM with background autofluorescence subtracted.
A. CD11b+Gr1- granulocytes from the spleen

B. IFN-γ expression on CD11b+Gr1- cells from the granulocyte region of the spleen

* p < 0.0054
Figure 48: MMC cells secrete GM-CSF, VEGF, and MCP-1

A multiplex cytokine array was performed on supernatants collected from MMC cultures after 24 hours; data are the average of duplicate samples.
Cytokine secretion by MMC cells

<table>
<thead>
<tr>
<th></th>
<th>mIL-1β</th>
<th>mIL-4</th>
<th>mIL-10</th>
<th>mIL-13</th>
<th>mIFN-γ</th>
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<tr>
<td>Secretion by MMC cells (pg/mL)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;10</td>
<td>&lt;4</td>
<td>&lt;1</td>
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of IL-6. Injections were done i.d. and were administered once per day for 3 consecutive
days, with the exception of high dose GM-CSF (2500ng), which was administered i.d. for
2 consecutive days. Twenty-four hours after the last injection, mice were sacrificed and
their spleens, bone marrow, and blood subjected to flow cytometry analysis for
CD11b+Gr1+ cells. Figure 49 shows representative data from the spleens of FVB mice
receiving various treatments. Representative forward scatter vs, side scatter plots show that
there is no difference in the overall proportion of the spleen that is made up of granulocytes
when comparing mice treated with PBS, mice treated with 500ng of GM-CSF, 2500ng of
GM-CSF, or 500ng GM-CSF with 120U IL-6 (Fig. 49A). Although the percentage of
MDSC in the granulocyte region varies moderately between mice, with the 500ng dose of
GM-CSF causing a 4% increase over the control and the GM-CSF with IL-6 treatment
causing a 9% increase over the control, this difference did not constitute a substantial
fraction of the whole spleen (Fig. 49B,C), nor was it recapitulated in calculating the
absolute number of MDSC in the spleen (Fig. 49D). Flow cytometry analysis of the bone
marrow showed that levels of MDSC in the four treatments groups were indeed very
similar. Figure 50A shows that the granulocyte region of the bone marrow in PBS-treated
mice comprised 39% of the total bone marrow, whereas the granulocyte regions of mice
receiving 500ng GM-CSF, 2500ng GM-CSF, or 500ng GM-CSF with 120U IL-6
comprised 39%, 41%, and 41% of the whole bone marrow, respectively. Furthermore, the
percentage of MDSC making up the granulocyte region was similar in all cases, with PBS-
treated mice having the highest percentage of MDSC (88.3%) as compared to 84% in the
500ng GM-CSF mouse, 79.6% in the 2500ng GM-CSF mouse, and 83.4% in the GM-CSF
Figure 49: GM-CSF/IL-6 does not increase MDSC in the spleens of FVB mice

(A) Representative flow cytometry plots showing forward scatter vs side scatter for identification of the percentage of granulocytes out of total spleen cells from FVB mice that received 3 consecutive daily intradermal injections of either PBS, GM-CSF (500ng), or GM-CSF (500ng) + IL-6 (120U), or 2 consecutive intradermal injections of GM-CSF (2500ng).

(B) Representative flow cytometry plots of CD11b+Gr1+ cells from the granulocyte region of the spleens of each of the four treatment groups described. (C) Representative flow cytometry plots of CD11b+Gr1+ cells from the whole spleen of each of the four treatment groups described. (D) Average absolute number of total MDSC in the spleens of each of the four treatment groups as determined by the total percentage of MDSC and trypan blue exclusion.
with IL-6 mouse (Fig 50B). These observations were similar when observing the percentage of MDSC that made up the whole bone marrow (Fig 50C) and was confirmed by similar absolute numbers of MDSC recovered from bone marrow in the tibias and femurs (Fig 50D). Flow cytometry of the peripheral blood also showed no discernable increase in MDSC in the granulocyte regions of mice treated with 500ng of GM-CSF (3.1%), 2500ng of GM-CSF (2.9%), or GM-CSF with IL-6 (4.8%) as compared to the PBS-treated controls (4.1%, Fig 51A). The same is true for MDSC levels in the granulocyte regions of these mice, as well the overall level of MDSC in the total peripheral blood (Fig 51B,C). Therefore neither GM-CSF nor GM-CSF in conjunction with IL-6 was able to increase MDSC in FVB mice after i.d. injection.

We next sought to determine if VEGF could be the MMC-derived soluble factor responsible for the increase in MDSC seen after injection of MMC-derived supernatant. We therefore treated naïve FVB mice with once-daily i.d. injections of 1.5µg of VEGF, or with PBS for 3 consecutive days. Mice were sacrificed on the fourth day and their spleens, bone marrow, and blood subjected to flow cytometry analysis. Representative data in Figure 52A shows that there was not a substantial increase in the granulocyte region of the spleen of VEGF-treated animals (4.9%) as compared to PBS-treated animals (4.2%). Likewise, there was no appreciable difference in the levels of MDSC within those granulocyte regions, with PBS-treated mice having 24.7% MDSC, while VEGF-treated mice had 26.4% MDSC (Fig. 52B). The percentage of MDSC in the whole spleen was also similar, with PBS-treated animals having 2.1% MDSC in their spleens, while VEGF-treated animals had 2.3% MDSC in their spleens (Fig. 52C).
Figure 50: GM-CSF/IL-6 does not increase MDSC in the bone marrow of FVB mice

(A) Representative flow cytometry plots showing forward scatter vs side scatter for identification of the percentage of granulocytes out of total bone marrow cells from FVB mice that received 3 consecutive daily intradermal injections of either PBS, GM-CSF (500ng), or GM-CSF (500ng) + IL-6 (120U), or 2 consecutive intradermal injections of 2500ng of GM-CSF. (B) Representative flow cytometry plots of CD11b+Gr1+ cells from the granulocyte region of the bone marrow of each of the four treatment groups described. (C) Representative flow cytometry plots of CD11b+Gr1+ cells from the total bone marrow of each of the four treatment groups described. (D) Number of MDSC in the bone marrow isolated from the tibias and femurs of each of the four treatment groups, as determined using the total percentage of MDSC and trypan blue exclusion.
Figure 51: GM-CSF/IL-6 does not increase MDSC in the blood of FVB mice
(A) Representative flow cytometry plots showing forward scatter vs side scatter for identification of the percentage of granulocytes out of total blood from FVB mice that received 3 consecutive daily intradermal injections of either PBS, GM-CSF(500ng), or GM-CSF(500ng) + IL-6 (120U), or 2 consecutive intradermal injections of 2500ng of GM-CSF.
(B) Representative flow cytometry plots of CD11b+Gr1+ cells from the granulocyte region of the blood of each of the four treatment groups described. (C) Representative flow cytometry plots of CD11b+Gr1+ cells from the total blood of each of the four treatment groups described.
Figure 52: Treatment with VEGF *in vivo* does not increase levels of MDSC in the spleens of FVB mice

FVB mice were treated with i.d. injection of 1.5μg of VEGF per day for 3 consecutive days or were treated with PBS. Mice were killed on the fourth day and flow cytometry was used to determine the levels of MDSC in the spleen. (A) Representative plots of forward scatter vs side scatter from the spleens of mice treated with VEGF or PBS for the quantification of granulocytes. (B) Percentage of CD11b+Gr1+ cells from within the granulocyte regions of mice treated with VEGF or PBS. (C) Percentage of CD11b+Gr1+ cells out of the whole spleen from mice treated with VEGF or PBS. (D) Absolute number of CD11b+Gr1+ cells in the spleens of mice treated with VEGF or PBS. Data are representative plots from 2-4 mice per group.
Calculation of the absolute number of MDSC in the spleens of each animal confirmed that there was no significant difference between the number of MDSC in the spleens of PBS-treated mice and VEGF-treated mice (Fig. 52D). The bone marrow of VEGF-treated mice also did not show any increase in MDSC as compared to PBS-treated controls.

Representative flow cytometry data in Figure 53A shows that the percentages of granulocytes in the two groups are nearly equal (35.4% in PBS-treated mice vs. 36.8% in VEGF-treated mice). Levels of MDSC within the granulocyte region were also similar, with MDSC making up 82.4% of the granulocytes in PBS-treated mice and 77.0% in VEGF-treated mice (Fig. 53B). These data were confirmed by the percentage of MDSC comprising the whole bone marrow (36.7% in PBS-treated mice and 33.4% in VEGF-treated mice, Fig. 53C) and the absolute number of MDSC in the bone marrow recovered from the tibias and femurs (7.5X10^6 MDSC in PBS-treated mice and 4.8X10^6 MDSC in VEGF-treated mice, Fig. 53D). Figure 54 shows representative data from the blood of PBS-treated and VEGF-treated mice. There was no difference in the percentage of granulocytes in the blood, with the blood of PBS-treated mice composed of 3% granulocytes while the blood of VEGF-treated mice was composed of 2% granulocytes (Fig. 54A). Although there seemed to be a large increase in the composition of the granulocyte region, with the control mice having 44% MDSC while VEGF mice had 66% MDSC, this difference was not statistically significant (Fig. 54B and data not shown). The percentage of MDSC in the entire population of peripheral blood leukocytes was similarly low in both groups (Fig. 54C).
Figure 53: Treatment with VEGF in vivo does not increase levels of MDSC in the bone marrow of FVB mice

FVB mice were treated with i.d. injection of 1.5 μg of VEGF per day for 3 consecutive days or were treated with PBS. Mice were killed on the fourth day and flow cytometry was used to determine the levels of MDSC in the spleen. (A) Representative plots of forward scatter vs side scatter from the bone marrow of mice treated with VEGF or PBS for the quantification of granulocytes. (B) Percentage of CD11b+Gr1+ cells from within the granulocyte regions of mice treated with VEGF or PBS. (C) Percentage of CD11b+Gr1+ cells out of the whole bone marrow from mice treated with VEGF or PBS. (D) Number of CD11b+Gr1+ cells in the bone marrow isolated from the tibias and femurs of mice treated with VEGF or PBS. Data are representative plots from 2-4 mice per group.
Figure S4: Treatment with VEGF \textit{in vivo} does not increase levels of MDSC in the blood of FVB mice

FVB mice were treated with i.d. injection of 1.5 μg of VEGF per day for 3 consecutive days or were treated with PBS. Mice were killed on the fourth day and flow cytometry was used to determine the levels of MDSC in the blood. (A) Representative plots of forward scatter vs side scatter from the blood of mice treated with VEGF or PBS for the quantification of granulocytes. (B) Percentage of CD11b+Gr1+ cells from within the granulocyte regions of mice treated with VEGF or PBS. (C) Percentage of CD11b+Gr1+ cells out of the whole blood from mice treated with VEGF or PBS.
Since injections of GM-CSF and VEGF failed to produce significant increases in MDSC \textit{in vivo}, we hypothesized that MCP-1 may play a role in the increase in MDSC caused by MMC-derived soluble factors. We therefore injected FVB mice i.d. with 2.5µg of MCP-1 or with PBS once per day for 3 consecutive days. Mice were sacrificed on the fourth day and their spleens, bone marrow, and blood were subjected to analysis by flow cytometry. Representative flow cytometry data in Figure 55A shows that the percentages of granulocytes in the spleen are similar between the two groups (5% in PBS-treated mice and 7% in MCP-1-treated mice). The percentage of MDSC in the granulocyte regions was also similar, with PBS-treated mice having 4% MDSC and MCP-1-treated mice having 7% MDSC (Fig 55B). Similar levels of total MDSC were also found in the spleen, with PBS-treated mice having 0.19% MDSC and MCP-1-treated mice having 0.40% MDSC (Fig. 55C). Calculation of the absolute numbers of MDSC in the spleens confirmed that there was no significant difference in MDSC in the spleens when comparing PBS-treated mice and MCP-1-treated mice (Fig 55D). The bone marrow of FVB mice treated with either PBS or MCP-1 also had very similar levels of granulocytes (29% in both groups), and MDSC within those granulocytes (78% in control mice and 80% in MCP-1-treated mice, Fig. 56A,B). The percentage of MDSC in the whole bone marrow was also similar between the two groups (22.9% in control mice and 23% in MCP-1-treated mice, Fig. 56C). The absolute number of MDSC in the bone marrow recovered from the tibias and femurs reflects the above data, with control mice having 7.51X10^6 MDSC and MCP-1 treated mice having 8.151X10^6 MDSC, a difference that was not statistically significant (Fig.56D). Flow cytometry of the blood of PBS-treated and MCP-1- treated mice showed that there
Figure 55: Treatment with MCP-1 \textit{in vivo} does not increase levels of MDSC in the spleens of FVB mice

FVB mice were treated with i.d. injection of 2.5\,$\mu$g of MCP-1 per day for 3 consecutive days or were treated with PBS. Mice were killed on the fourth day and flow cytometry was used to determine the levels of MDSC in the spleen. (A) Representative plots of forward scatter vs side scatter from the spleens of mice treated with MCP-1 or PBS for the quantification of granulocytes. (B) Percentage of CD11b+Gr1+ cells from within the granulocyte regions of mice treated with MCP-1 or PBS. (C) Percentage of CD11b+Gr1+ cells out of the whole spleen from mice treated with MCP-1 or PBS. (D) Absolute number of CD11b+Gr1+ cells in the spleens of mice treated with MCP-1 or PBS. Data are representative plots from 2-4 mice per group.
Figure 56: Treatment with MCP-1 \textit{in vivo} does not increase levels of MDSC in the bone marrow of FVB mice.

FVB mice were treated with i.d. injection of 2.5 \textmu g of MCP-1 per day for 3 consecutive days or were treated with PBS. Mice were killed on the fourth day and flow cytometry was used to determine the levels of MDSC in the bone marrow. (A) Representative plots of forward scatter vs side scatter from the bone marrow of mice treated with MCP-1 or PBS for the quantification of granulocytes. (B) Percentage of CD11b+Gr1+ cells from within the granulocyte regions of mice treated with MCP-1 or PBS. (C) Percentage of CD11b+Gr1+ cells out of the whole bone marrow from mice treated with MCP-1 or PBS. (D) Number of CD11b+Gr1+ cells in the bone marrow isolated from the tibias and femurs of mice treated with MCP-1 or PBS. Data are representative plots from 2-4 mice per group.
Figure 57: Treatment with MCP-1 *in vivo* does not increase levels of MDSC in the blood of FVB mice

FVB mice were treated with i.d. injection of 2.5 μg of MCP-1 per day for 3 consecutive days or were treated with PBS. Mice were killed on the fourth day and flow cytometry was used to determine the levels of MDSC in the blood. (A) Representative plots of forward scatter vs side scatter from the blood of mice treated with MCP-1 or PBS for the quantification of granulocytes. (B) Percentage of CD11b+Gr1+ cells from within the granulocyte regions of mice treated with MCP-1 or PBS. (C) Percentage of CD11b+Gr1+ cells out of the whole blood from mice treated with MCP-1 or PBS. Data are representative plots from 2-4 mice per group.
was not increase in the granulocyte regions of MCP-1-treated mice (2%) over PBS-treated mice (3%, Fig. 57A). There was an increase in MDSC within the granulocyte region, however this difference was not found to be statistically significant (Fig 57B and data not shown). The percentage of the total peripheral blood leukocytes made up of MDSC was again very similar between the groups with no increase seen in mice treated with MCP-1 (0.75%) as compared to mice treated with PBS (0.9%, Fig. 57C).

**GM-CSF is Responsible for the Generation and Maintenance of MDSC**

Considering the lack of responses to cytokines/chemokines that were found to be secreted by MMC and were injected in vivo, we decided to look at responses to these cytokines in vitro. Since GM-CSF is a well-established adjuvant therapy for the differentiation of dendritic cells in vivo, (244, 245) we wanted to determine if GM-CSF had any effect specifically on MDSC. Bone marrow was therefore isolated from naïve FVBN202 mice and was cultured for 6 days in the presence of 100ng/mL of GM-CSF. Flow cytometry staining was done for CD11b and Gr1 on day 6. As shown in Figure 58A, 77% of the bone marrow of FVBN202 mice is comprised of CD11b+Gr1+ cells on day 0, while 5% of the cells are CD11b-Gr1+, 4% of the cells are CD11b+Gr1-, and 14% of the cells are CD11b-Gr1- undifferentiated progenitor cells. In contrast, on day 6, only 25.1% of the bone marrow cells remained as CD11b+Gr1+, while the CD11b-Gr1+ population had decreased to 0.6% and the population of progenitor cells had decreased to 1.8%. However, 72.6% of the cells on day 6 were CD11b+Gr1- cells, a phenotype that is
Figure 58: Culture of whole bone marrow with GM-CSF results in an increase in CD11b+Gr1- cells

(A) Bone marrow cells from tumor free FVBN202 mice were harvested and stained on day 0 for CD11b and Gr1. Bone marrow cells were cultured for 6 days with 100ng/mL GM-CSF and media was changed every other day. Cells were stained again in day 6 for the expression of CD11b and Gr1. Analyses are of the total viable cells and are representative of 3 separate experiments. (B) Number of CD11b+Gr1+ cells present in the bone marrow recovered from the tibias and femurs of FVBN202 mice on day 0 as compared to the absolute number of CD11b+Gr1+ cells after 6 days of culture with GM-CSF, as above. Data are averages of 3 separate experiments +/- SEM.
indicative of mature dendritic cells and macrophages. GM-CSF may therefore cause the
differentiation of CD11b+Gr1+ cells into CD11b+Gr1- cells, the expansion of
CD11b+Gr1- cells, or the differentiation of CD11b-Gr1- progenitor cells. Because the
absolute number of CD11b+Gr1+ cells actually increased on day 6 as compared to day 0
(6.0x10^6 MDSC on day 0 as compared to 10.9x10^6 MDSC on day 6, Fig. 58B), we
hypothesized that instead of maturing these cells, that GM-CSF was actually maintaining a
population of these cells, and even causing the generation of more MDSC. We also wanted
to determine if a 6 day culture with GM-CSF affected the differentiation/ migration status
of MDSC. Flow cytometry was therefore done on day 6 for CD11b, Gr1, and CD86 as well
as CD11b, Gr1, and CCR7. Figure 59A shows that the maturation marker CD86 was
greatly increased from being expressed on 22.7% of MDSC on day 0 to being expressed on
58.3% of MDSC on day 6 (p=0.008). Furthermore, CCR7, a chemokine receptor that has
been demonstrated to be essential for lymphocyte homing to peripheral lymphoid organs
(246, 247) and expression of which has been correlated with dendritic cell maturation
(248) was observed to be down-regulated after 6 days of culture with GM-CSF (58.4% of
MDSC expressed CCR7 on day 0 and 35.9% of MDSC expressed CCR7 on day 6,
P=0.00078, Fig. 59B). This suggests that GM-CSF, which is secreted by MMC cells, may
have a direct effect on MDSC and affect their generation and/or maintenance in the
periphery.

In order to further test the effect that GM-CSF may be having specifically on
MDSC or MDSC progenitors and to determine which MMC-derived cytokine was
necessary for the increased production of MDSC, bone marrow cells from naïve FVBN202
Figure 59: Culture of whole bone marrow with GM-CSF causes an increase in CD86 expression and a decrease in CCR7 expression.

(A) Bone marrow was isolated from naïve FVB/N202 mice and was cultured for 6 days in 100ng/mL of GM-CSF; media was changed every other day. Staining was done on day 0 and again on day 6 after culture for CD11b, Gr1, and CD86. Representative plots of 3 separate experiments display the percentage of gated CD11b+Gr1+ cells from the granulocyte region that express CD86 (black line) as compared to background (autofluorescence) for that sample (gray) for day 0 and day 6. Bar graph shows the average of this data from 3 separate experiments +/- SEM with the autofluorescence subtracted.

(B) Bone marrow was cultured as above. Staining was done on day 0 and day 6 for CD11b, Gr1, and CCR7. Representative plots from 2 separate experiments shows the percentage of gated CD11b+Gr1+ cells from the granulocyte region that express CCR7 (black line) as compared to background (autofluorescence) for that sample (gray) for day 0 and day 6. Bar graph shows the average of 2 experiments +/- SEM with autofluorescence subtracted.
A. Bone marrow from naïve FVB/N202 mouse

Day 0

CD86

Day 6

CD86 expression on MDSC

Percentage

Day 0

Day 6

*p=0.008

B. Bone marrow from naïve FVB/N202 mouse

Day 0

CCR7

Day 6

CCR7 expression on MDSC

Percentage

Day 0

Day 6

*p=0.00078
mice were harvested, stained with antibodies against CD11b and Gr1, and were sorted into 2 populations: CD11b+Gr1+ cells and CD11b-Gr1- cells. The purity of the sorted cells was >96% (Fig. 60A). Sorted cells were cultured separately in the presence of GM-CSF (100ng/mL), VEGF (50ng/mL), MCP-1 (50ng/mL), M-CSF (50ng/mL), G-CSF (50ng/mL) or MMC cells on top of a Transwell insert. Control wells contained medium only. Media was changed on day 3 and fresh cytokine or tumor cells added where applicable. On day 6, cells were subjected to flow cytometry analysis to determine generation of MDSC from CD11b-Gr1- progenitor cells or maintenance of existing CD11b+Gr1+ MDSC in vitro. Figure 60B shows that MMC-derived soluble factors or GM-CSF caused the generation of MDSC from hematopoietic CD11b-Gr1- cells while the presence of media resulted in a negligible effect. At the end of a 6-day culture 46.9% and 56.8% of cells were MDSC in the presence of MMC (P=0.007 as compared to media alone) and GM-CSF (P=0.001 as compared to media alone), respectively. A Similar observation was made when GM-CSF was used at a lower concentration of 10ng/mL (data not shown). VEGF or MCP-1, M-CSF, and G-CSF did not induce generation of MDSC from CD11b-Gr1- cells (data not shown). Figure 60C shows that sorted CD11b+Gr1+ MDSC exhibited a significant drop in CD11b+Gr1+ levels from day 0 in all culture conditions. However, both GM-CSF (61.0%) and MMC (42.5%), supported the maintenance of CD11b+Gr1+ cells when compared with media alone (10.5%) (P=0.013 and P=0.006, respectively). Each cytokine/chemokine was also tested for the induction of proliferation in sorted CD11b+Gr1+ cells, and it was determined that none could induce proliferation of MDSC exceeding the baseline level (data not shown). Therefore, GM-CSF was found to be the major cytokine involved in the
Figure 60: MMC-derived soluble factors and GM-CSF can generate MDSC from CD11b-Gr1- progenitor cells, and can maintain existing MDSC.

Bone marrow cells from naïve FVBN202 mice were stained and sorted into 2 populations: CD11b+Gr1+ and CD11b-Gr1- cells. (A) Flow cytometry plots of the purity of CD11b+Gr1+ and CD11b-Gr1- populations after sorting. Data are representative of duplicate experiments. (B) Representative flow cytometry plots showing the percentage of CD11b+Gr1+ cells 6 days after culture of sorted CD11b-Gr1- cells (left) and averages of 3-4 experiments (right). (C) Representative flow cytometry plots showing the percentage of CD11b+Gr1+ cells remaining on day 6 after culture of sorted CD11b+Gr1+ cells (left) and averaged data from 3-4 experiments (right).
A. Sorted CD11b-Gr1- cells  Sorted CD11b+Gr1+ cells

B. CD11b-Gr1-

Media  MMC  GM-CSF

CD11b+Gr1+ cells after a 6-day culture of BM-derived CD11b-Gr1- cells

C. CD11b+Gr1+

Media  MMC  GM-CSF

CD11b+Gr1+ cells after a 6-day culture of BM-derived MDSC
conversion of CD11b-Gr1- cells to MDSC as well as maintenance, but not proliferation of existing MDSC.

Since one of the characteristics of MDSC is their short half-life and high turnover rates, (216) we sought to determine if any of the MMC-derived soluble factors could inhibit apoptosis of newly arising MDSC or existing MDSC. Bone marrow cells were harvested as before and were sorted into CD11b+Gr1+ and CD11b-Gr1- populations. Cells were stained with Annexin V after sorting to determine the viability of each population just before culture. The double negative and double positive sorted populations contained 63.0% and 36.8% Annexin V positive apoptotic cells, respectively (representative data of duplicate experiments are shown in Fig. 61A). Cells were then cultured in conditions as described for Figure 60 and were stained on day 6 with antibodies against CD11b and Gr1, and with Annexin V. Figure 61B shows levels of apoptosis in the newly converted MDSC. GM-CSF significantly reduced the percentage of apoptotic MDSC as compared to the proportion of apoptotic cells in the presence of media alone (18.7% vs. 92.3%, P=0.00005). Annexin V levels were also reduced in MDSC cultured with MMC in Transwell as compared to those cultured in media alone (58.3% vs. 92.3%, P=0.024). Interestingly, apoptosis was significantly reduced in MDSC when progenitor CD11b-Gr1- cells were cultured with GM-CSF as compared to those cells cultured with MMC in transwell (18.7% vs 58.3%, P=0.003). Figure 61C shows levels of apoptosis in previously generated MDSC after a 6-day culture in vitro. The CD11b+Gr1+ sorted cells exhibited significantly reduced apoptosis when cultured with GM-CSF as compared to media alone (38.8% vs. 91.1%, P=0.0001). Annexin V positive MDSC were significantly reduced in
Sorted populations of CD11b+Gr1+ or CD11b-Gr1- cells were cultured for 6 days with media, MMC, or GM-CSF and stained on day 6 for CD11b, Gr1, and Annexin V. (A) Representative flow cytometry plots of duplicate experiments showing the expression of Annexin V on sorted CD11b+Gr1+ cells (left) or sorted CD11b-Gr1- cells (right) just after sorting. (B) Representative flow cytometry plots of CD11b+Gr1+ cells after 6 days of culture of sorted CD11b-Gr1- cells (boxed region) that are Annexin V positive. Bar graph shows the average percentage of CD11b+Gr1+AnnexinV+ cells from 3-4 experiments. (C) Representative flow cytometry plots of CD11b+Gr1+ after 6 days of culture of sorted CD11b+Gr1+ cells (boxed region) that are Annexin V positive. Bar graph depicts the averages of 3-4 experiments.
Day 0

A. Sorted CD11b+Gr1+ Cells vs Sorted CD11b-Gr1- Cells

Day 6 CD11b-Gr1-

B. Media, MMC, GM-CSF

Annexin+CD11b+Gr1+ cells after a 6 Day culture of double negative bone marrow precursors

Day 6 CD11b+Gr1+

C. Media, MMC, GM-CSF

Annexin+CD11b+Gr1+ cells after a 6 day culture of sorted MDSC
the presence of MMC in Transwell as compared to media alone (65.0% vs. 91.1%, \( P=0.001 \)). Apoptosis was also significantly reduced when comparing Annexin V positive MDSC in the presence of GM-CSF compared to those cultured with MMC in Transwell (38.8% vs 65.0%, \( P=0.003 \)). Levels of apoptosis in CD11b+Gr1+ cells on day 0 (Fig. 61A) were similar to those seen on day 6 after culture with GM-CSF (Fig. 61C) (36.8% on day 0 vs. 38.8% on day 6), whereas CD11b-Gr1- cells cultured with GM-CSF showed decreased Annexin V levels on day 6 (Fig. 61B) compared to day 0 (Fig. 61A) (63.0% on day 0 vs 18.7% on day 6, \( P=0.017 \)). Therefore, GM-CSF seems to augment the survival of early stage MDSC as compared to late stage MDSC. Annexin V staining on CD11b+Gr1+ cells, both newly converted and existing, cultured with VEGF, MCP-1, or G-CSF were similar to Annexin V levels on MDSC cultured in media alone (data not shown).

**FVBN202 Mice Display Unique Subsets of MDSC with GM-CSF Selectively Driving the Generation of the Suppressive Ly6G-Ly6C+ Subset**

It has recently been reported that various subsets of MDSC exist and can be distinguished based on their differential expression of two epitopes of the Gr1 molecule; Ly6G, corresponding to a granulocytic/neutrophil phenotype, and Ly6C, corresponding to a monocytic phenotype (171, 215, 216). We report here that our model of neu+ tumor-bearing FVBN202 mice, which has yet to be investigated, displays novel phenotypes and characteristics of MDSC. Two main subsets exist in FVBN202 mice, Ly6G-Ly6C\textsuperscript{low/int}, and Ly6G+Ly6C\textsuperscript{high}, with the former displaying suppressive capabilities while the latter showed no suppression against CD3/CD28 stimulated T cells (Fig. 35). Previously
reported subsets include Ly6G-Ly6C\textsuperscript{high}, Ly6G\textsuperscript{+}/Ly6C\textsuperscript{low/int}, and Ly6G\textsuperscript{int/dull}/Ly6C\textsuperscript{hi} (171, 215, 216). These phenotypes display an inverse correlation between Ly6G and Ly6C expression, where high Ly6G expression is associated with intermediate or low Ly6C expression and low or absent Ly6G expression is associated with high Ly6C expression, which is in direct contrast to the phenotypes we present here. Furthermore, reports have differed in regards to which subset exhibits greater expansion and/or suppressive function.

Figure 62A shows CD11b\textsuperscript{+} cells from the spleens of tumor-free and tumor-bearing FVBN202 mice, both of which display a smaller proportion of Ly6G-Ly6C\textsuperscript{int} (17% in tumor-free and 16% in tumor-bearing) and a much larger proportion of Ly6G+Ly6C\textsuperscript{high} cells (24% in tumor-free and 83% in tumor-bearing). Figure 62C shows a significant expansion of both the Ly6G-Ly6C\textsuperscript{int} and Ly6G+Ly6C\textsuperscript{high} fractions (P=0.008 and 0.003, respectively) in the whole spleens of tumor bearing mice as compared to tumor free mice. Similar phenotypes and proportions of cells are seen in the bone marrow. As shown in Figure 62B, tumor-free mice contain a population of Ly6G-Ly6C\textsuperscript{low} cells, with a lower MFI than that seen in the spleen or in the bone marrow of tumor-bearing animals. Like Ly6G-Ly6C\textsuperscript{int} cells, Ly6G-Ly6C\textsuperscript{low} cells are found at a lower proportion (19%) than Ly6G+Ly6C\textsuperscript{high} cells (80%) in the bone marrow of tumor-free mice. Although these proportions are similar in the bone marrow of tumor-bearing animals, the expression of Ly6C per cell increases on the Ly6G- fraction, creating a population of Ly6G-Ly6C\textsuperscript{int} cells as seen in the spleen (Fig 62B). Figure 62D shows significant expansion of both the Ly6G-Ly6C\textsuperscript{low/int} population (P=0.002) and the Ly6G+Ly6C\textsuperscript{high} population (P=0.001) in the whole bone marrow of tumor-bearing animals as compared to tumor-free animals. It is
Figure 62: FVBN202 mice contain unique subsets of MDSC

Spleen cells (A) or bone marrow cells (B) were isolated from tumor-free (TF) or tumor-bearing (TB) FVBN202 mice and stained for CD11b, Ly6G, and Ly6C and analyzed by flow cytometry. Results are representative of 3 mice per group and display percentages of cells as gated on CD11b+ cells from within the granulocyte region. (C) Composition of subsets in the whole spleens of TB vs TF mice. Data are averages of 3 mice per group +/- SEM. (D) Composition of subsets in the whole bone marrow in TB vs TF mice. Data are averages of 3 mice per group +/- SEM.
noteworthy that both fractions expand similarly in the bone marrow (3.2 fold expansion of the Ly6G-Ly6C\textsuperscript{low/int} fraction and 3.1 fold expansion of the Ly6G+Ly6C\textsuperscript{high} fraction), while expansion in the spleens of tumor-bearing mice showed a 23.6-fold increase in Ly6G-Ly6C\textsuperscript{low/int} MDSC as compared to splenocytes from tumor free mice (P=0.008), whereas the Ly6G+Ly6C\textsuperscript{high} fraction showed a drastic 64.2-fold increase in tumor bearing spleens (P=0.003).

Given the presence of these unique subsets and the fact that GM-CSF and MMC-derived soluble factors induced the generation of MDSC from CD11b-Gr1- cells (Fig. 60), we next sought to determine the effects of GM-CSF and MMC on MDSC subsets. Bone marrow cells were therefore sorted from tumor-free FVBN202 mice as before and cultured in the presence of GM-CSF, MMC in Transwell, or media alone for 6 days. Flow cytometry was performed on day 0 and day 6 to analyze the expression of Ly6G and Ly6C. As shown in Figure 62B, MDSC in the bone marrow of tumor free mice on day 0 were comprised of 19% Ly6G-Ly6C+, correlating with a suppressive phenotype and 80% Ly6G+Ly6C+ correlating with non-suppressive phenotype. Interestingly, culture of the progenitor cells in the presence of MMC or GM-CSF resulted in a significant increase in the suppressive Ly6G-Ly6C+ subset as compared to day 0 (58% with MMC, \(P=0.018\) and 72% with GM-CSF, \(P=0.015\), Fig. 63A). Furthermore, the amount of suppressive cells generated from culture with GM-CSF was significantly higher than that generated from culture with MMC in Transwell (Fig. 63A: 72% with GM-CSF and 58% with MMC, \(P=0.045\)). It is noteworthy here that the mean fluorescent intensity (MFI) of Ly6C was increased substantially in the Ly6G- fraction after culture with GM-CSF, and even more so
after culture with MMC, as compared to levels seen in tumor free bone marrow on day 0 (Fig. 62B). This is consistent with an increase in Ly6C MFI that we have observed in the bone marrow of tumor-bearing mice when compared to tumor free mice (Fig. 62B).

Residual MDSC in the presence of medium alone showed a negligible percent of viable cells of either population (data not shown). Figure 63B shows that culture of CD11b+Gr1+ cells with MMC maintained a proportion of Ly6G-Ly6C_{low/int} cells similar to that on day 0 (17% vs. 19%), while the percentage of Ly6G+Ly6C_{high} non-suppressive cells decreased significantly from day 0 (47% vs. 80%, \( P=0.001 \)). Culture of sorted CD11b+Gr1+ cells with GM-CSF, however, resulted in a significantly reduced percentage of Ly6G-Ly6C_{low/int} cells (8% vs. 19%, \( P=0.011 \)) as well as a significant reduction in Ly6G+Ly6C_{high} cells (41% vs. 80%, \( P=0.0004 \), Fig. 63B). Again, MDSC in the presence of medium alone showed negligible percent of viable Ly6G-Ly6C_{low/int} or Ly6G+Ly6C_{high} cells (data not shown).

We next wanted to determine if culture of bone marrow progenitor cells with MMC in Transwell or with GM-CSF could affect the status of MHC class I and MHC class II molecules on MDSC, as we have observed previously (Figs. 46,47). Sorted CD11b-Gr1-bone marrow cells from tumor free FVBN202 mice were therefore cultured for 6 days as before. Flow cytometry analysis on day 6 shows that culture with MMC or GM-CSF causes the downregulation of H-2\(^d\) on the surface of MDSC. Representative data in Figure 64A shows that, of MDSC cultured in media alone, 72.3% expressed H-2\(^d\) as compared to 18.6% and 26.5% of newly-derived MDSC expressing H-2\(^d\) after culture with MMC and
Figure 63: MMC-derived supernatant and GM-CSF cause the generation of suppressive CD11b+Ly6G-Ly6C+ MDSC from CD11b-Gr1- precursor cells

(A) MDSC from tumor-free FVB/N202 mice were analyzed on day 0 for the expression of Ly6G and Ly6C by flow cytometry. CD11b-Gr1- cells sorted from these mice were analyzed on day 6 for expression of Ly6G and Ly6C by flow cytometry. Bar graph shows the average of duplicate wells cultured with MMC cells or GM-CSF and 3 naïve mice on day 0. (B) CD11b+Gr1+ cells were sorted from naïve FVB/N202 mice and were cultured for 6 days as previously. The expression of Ly6G and Ly6C was analyzed on CD11b+ cells on day 6 (average of duplicate wells) and is compared to levels seen in tumor free FVB/N202 mice on day 0 (average of 3 mice +/- SEM).
GM-CSF, respectively (Fig 64A). These data differed slightly after subtraction of background levels of autofluorescence, with H-2\(^q\) expression on MDSC after culture in GM-CSF being lower than that seen on MDSC after culture with MMC, however this was not a significant difference. On the other hand, both of these adjusted values are significantly lower than those seen in media alone (P=0.003 and P=1.9\times10^{-5} in comparing media to MMC and GM-CSF cultures, respectively). Likewise, the expression of the MHC class II molecule I-A\(^q\) was also reduced after culture with both MMC and GM-CSF. Culture with media alone resulted in 54.9% of MDSC expressing I-A\(^q\), while culture with MMC reduced this number to 14.7% and culture with GM-CSF resulted in 24.2% of MDSC expressing I-A\(^q\) (Fig. 64B top row). As previously, subtraction of background autofluorescence yielded even more substantial differences between the cells cultured in media and those cultured with MMC (P=0.005 as compared to media alone) or GM-CSF (P=0.0098 as compared to media alone) (Fig. 64B bottom graph). We further wanted to elucidate if existing MDSC are able to proliferate in response to GM-CSF or MMC-derived soluble factors \textit{in vitro}. To clarify this point, sorted CD11b+Gr1+ cells from the bone marrow of tumor free mice were pulsed with BrdU on day 0 of the culture period and then were cultured for 3 days with media alone, MMC on a Transwell insert, or 100ng/mL of GM-CSF. On day 3, cells were harvested and strained for CD11b and Gr1 as well as for the intracellular incorporation of BrdU. Figure 65A shows the change in the percentage of BrdU+ CD11b+Gr1+ cells cultured with MMC or GM-CSF as compared to media alone. The increase in BrdU incorporation over baseline was less than 10% in both cases and was not statistically significant. This is recapitulated by the absolute cell numbers in Figure
64B. As indicated by the diamonds, the starting cell number on day 0 was $1.5 \times 10^5$ cells. The bars under the diamonds indicate the number of viable cells remaining on day 6. There was no proliferation seen in any of the cultures as assessed by absolute cell numbers (Fig. 65B).
Figure 64: Culture of CD11b-Gr1- cells with GM-CSF or MMC leads to a decrease in the expression of MHC class I and MHC class II molecules

(A) CD11b-Gr1- cells were sorted from naïve FVB/N202 bone marrow and cultured for 6 days as before. Cells were then analyzed by flow cytometry for the expression of H-2k on CD11b+Gr1+ cells. Plots are representative of 3 experiments and depict H-2k expression (black line) on day 6 as compared to the level of autofluorescence in each sample on day 6 (gray). Bar graph represents the average of 3 separate experiments +/- SEM with autofluorescence subtracted. (B) CD11b-Gr1- cells were sorted and cultured as above, on day 6, flow cytometry was done to analyze the expression of I-A\(^d\) on CD11b+Gr1+ cells. Plots are representative of 3 experiments and depict I-A\(^d\) expression (black line) on day 6 as compared to the level of autofluorescence in each sample on day 6 (gray). Bar graph represents the average of 3 separate experiments +/- SEM with autofluorescence subtracted.
A. MMC soluble factors and GM-CSF suppress the expression of H-2^d on newly converted MDSC.

B. MMC soluble factors and GM-CSF suppress the expression of I-A^d on newly converted MDSC.
Figure 65: GM-CSF and MMC do not cause the proliferation of sorted CD11b+Gr1+ cells in vitro.

(A) CD11b+Gr1+ cells were sorted from naïve FVB/N202 mice as before. Cells were pulsed with BrdU on day 0 of the culture period and then cultured as previously. On day 3 flow cytometry was done to analyze the uptake of BrdU into CD11b+Gr1+ cells. Data represent the percent of BrdU+ CD11b+Gr1+ cells present above the baseline level of proliferation seen with media alone. Data are averages of duplicate wells +/- SD. (B) Absolute cell number of CD11b+Gr1+ cells on day 6 after culture of sorted CD11b+Gr1+ cells in media alone, with GM-CSF, or with MMC. Data are averages of duplicate wells +/- SD.
A. Change in percentage of BrdU positive cells over medium alone

B. Change in absolute cell number on day 3 vs day 0
III. Conclusions and Significance

These studies clearly demonstrate that MMC-derived soluble factors do, in fact, cause the increase in MDSC in the spleen, blood, and bone marrow of FVBN202 mice. Furthermore, MHC class II expression was decreased on both CD11b+ and Gr1+ cells and CD11b+Gr1- cells. Cell sorting and subsequent culture with one of the predominant cytokines or chemokines secreted by MMC showed that GM-CSF, but not VEGF, IL-6, or MCP-1, specifically supported the maintenance of existing MDSC and the generation of new MDSC. GM-CSF was also increased the viability in both cell populations, although CD11b-Gr1- cells were more highly affected by GM-CSF than were CD11b+Gr1+ cells. CD11b+Gr1+ cells were not observed to proliferate in responses to GM-CSF; we therefore conclude that increases in MDSC can result from both the generation of new MDSC from bone marrow progenitor cells and the maintenance and survival of existing MDSC, but not from proliferation of MDSC, in response to GM-CSF. Strikingly, GM-CSF was found to specifically increase the suppressive CD11b+Ly6G-Ly6C+ phenotype of MDSC while decreasing the non-suppressive population arising from sorted CD11b-Gr1- progenitor cells, a trend not recapitulated with culture of existing MDSC. Exposure to GM-CSF also resulted in significant decreases of MHC class I and MHC class II on the surface of newly converted MDSC, an effect not seen with culture of existing MDSC. Therefore, exposure to GM-CSF at an early stage of differentiation can profoundly affect the resultant cellular profile, with increases in CD11b+Ly6G-Ly6C+ MDSC as well as sustained decreases in MHC class I and class II molecules, resulting in suppressive populations of granulocytes that do not express molecules for antigen presentation.
These results offer significant findings as to the mechanisms of MDSC accumulation and identify GM-CSF as a major tumor-derived soluble factor responsible for these effects which is of substantial clinical important given that many tumor vaccines use GM-CSF as an adjuvant and most human tumors also secrete GM-CSF, which has been correlated with increased levels of circulating myeloid cells (210, 244, 249). We propose that the use of GM-CSF as an adjuvant, in combination with tumor-derived GM-CSF, may elevate overall serum levels to a detrimental level and result in increases in MDSC in vivo as suggested by the finding that peptide vaccination coupled with GM-CSF in melanoma patients caused an increase in MDSC that was not observed in patients receiving peptide vaccination alone (213). Furthermore, neutralization of GM-CSF in patients with elevated serum levels may offer clinical benefit in conjunction with other immunotherapies.
Discussion

I. Chapter 1

Neu+ tumors in FVBN202 Mice Cause an Increase in MDSC that Prevents Successful Adoptive Immunotherapy

We have observed significant increases in granulocytes in the spleens, bone marrow, and blood of FVBN202 mice bearing MMC tumors, and found that the absolute number of granulocytes in these mice was significantly increased as well. These observations were concurrent with a significant decrease in the absolute number of cells in the lymphocyte region from the bone marrow of the tibias and femurs of tumor-bearing mice. This indicates that granulocytic precursors are able to overtake or out compete lymphocytic precursors in the bone marrow, or that lymphocytic precursors in the bone marrow may be more sensitive to inhibition by MDSC than mature lymphocytes in the spleen, where no significant decrease in absolute number was observed. This population of granulocytes in tumor-bearing mice was found to be mainly comprised of CD11b+Gr1+ cells and we found a dramatic increase in these cells in both the total population and within the granulocyte population in the spleen, bone marrow, and blood. The increase in MDSC in the spleens of TB mice is also associated with a decrease in CD11b single positive cells, although this phenomenon is not seen in the bone marrow. There is also a decrease in
CD11b+ cells in the granulocyte region of the blood, showing that although similar numbers of CD11b+ cells are present in the BM of tumor bearing and naïve mice, fewer of these cells are making it to the periphery. This increase in CD11b+Gr1+ cells and decrease in CD11b+ cells results in a decreased APC to T cell ratio, thus hindering antigen presentation in vivo. Given the prevalence of these cells in tumor-bearing mice, we hypothesized that these cells could be responsible for the failure of adoptively transferred neu-specific T cells to reject neu-positive mammary carcinomas.

Although the adoptive transfer of tumor-specific T cells that have been activated and expanded in vitro is a promising means of systemically treating residual cancers after resection of the primary tumor, the presence of immune suppressor cells may be a substantial obstacle to the success of these treatments. In particular, CD11b+Gr1+ MDSC represent a potent population of suppressor cells that are elevated in many different types of cancer and have been associated with suppression of T cell responses by multiple mechanisms.

To test our hypothesis, T cells from donor FVB mice donor mice that had rejected a challenge with neu+ MMC cells were activated with bryostatin and ionomycin and expanded in IL-2 for subsequent adoptive transfer. Although activation and expansion of these cells resulted in activated CD44+CD62L- and CD44+CD62L+ effector and memory T cells that showed cytotoxic effects against MMC in vitro, the transfer of these T cells offered no protection against MMC tumors in FVBN202 mice. In contrast, the profound inhibition of MMC tumors that we observed in mice that were given AIT in conjunction
with MDSC depletion confirmed the hypothesis that elevated MDSC can result in the failure of AIT. Importantly, the level of MDSC remaining in tumor-bearing mice was inversely correlated with the ability to reject tumors, showing the importance of effectively depleting MDSC in vivo. Furthermore, AIT coupled with MDSC depletion resulted in a robust antibody response generated against the extracellular domain of neu, which was not seen in the control group or the group receiving AIT alone. The presence of higher percentages of activated (CD25+) CD4+ and CD8+ T cells in the spleens of mice that received AIT alone as compared to both control mice and mice that received MDSC depletion is indicative of the a continued response by transferred T cells against MMC tumors in these mice. Mice that received MDSC depletion and exhibited tumor regression showed substantially lower levels of activated T cells most likely because these cells had undergone the contraction phase of the immune response, making the percentage of activated T cells similar to that seen in mice not receiving adoptive transfer. Therefore, the depletion of MDSC in vivo results in an anti-neu immune response which is capable of rejecting neu+ mammary tumors and tumor rejection requires both MDSC depletion and the presence of anti-tumor effector T cells, given that depletion of MDSC alone offered no protection against neu+ tumors.

**T cell Expansion Using Alternating Gamma Chain Cytokines Results in Greater Expansion, Cytotoxicity, and Viability**

In an effort to generate highly aggressive T cells that may overcome the suppressive effects of MDSC, we compared the phenotypes of T cells after expansion with
either IL-2 alone, IL-7/IL-15 alone, or with all three cytokines, called “alternating” cytokine expansion. We found expansion in alternating gamma chain cytokine conditions to be more effective than IL-2 alone or IL-7/IL-15 alone for the generation of large numbers of neu-specific anti-tumor effector T cells. Having compared IL-2 and IL-7/IL-15 with alternating gamma chain cytokines, we found that the alternating cytokine expansion was superior to expansion in IL-2 and equal to expansion in IL-7/IL-15 in terms of generating highly viable T cells. This is likely because IL-2 is known to up-regulate Fas/FasL and cause activation induced cell death (141) as well as the fact that IL-7 and IL-15 are important for the maintenance of memory T cells (130, 146, 223, 250, 251). Thus, cultures containing exogenous IL-7/IL-15 had more viable cells than cultures with IL-2 alone. Along with increased viability, cultures with added IL-7/IL-15 had more CD8+ T cells, with the proportions of CD4+ and CD8+ T cells being roughly equal, whereas cultures expanded in IL-2 alone showed fewer CD8+ T cells and a higher proportion of CD4+ T cells. IL-15 had been shown to be an important growth factor and activator of CD8+ T cells, as well as being shown to enhance the anti-tumor effects of CD8+ T cells. Furthermore, IL-7 and IL-15 in conjunction have been shown to be imperative for the homeostatic proliferation of memory CD8+ T cells (131, 148, 252). In addition to increased overall CD8+ T cells, the important role of IL-7/IL-15 in the generation and maintenance of memory T cells is also apparent. Expansion with IL-7/15 alone elicited 2 distinct populations of memory cells: CD62L^{low}CD44^{high} effector memory T cells and CD62L^{high}CD44^{low} central memory T cells, while expansion with IL-2 or with alternating cytokines both resulted in only T cells of the effector memory phenotype. This implies that
the addition of exogenous IL-2 for even a short period results in increased proportions of cells with effector-like phenotypes. This is further confirmed by the fact that expansion in IL-7/15 alone elicited substantially decreased levels of CD62L-CD44+ effector cells than either IL-2 or alternating cytokine expanded cells. Concurrent with the fact that alternating cytokine expansion resulted in the highest percentage of CD62L-CD44+ T cells, these cells showed a higher anti-tumor efficacy than IL-2-expanded T cells in vitro, as evaluated by cytotoxicity assays.

The markedly reduced expansion levels seen in cultures using IL-7/15 are a result of a lack of expression of the IL-2Rα chain (CD25) and the IL-7Rα chain and on both CD4+ and CD8+ T cells. IL-2 is a necessary T cell growth factor that is secreted by activated T cells and can work in an autocrine or paracrine fashion; cells expanded in IL-7/15 alone are not able to expand in response to IL-2 being made in the culture (253). Furthermore, since CD25 expression is augmented by IL-2 itself, the low levels of CD25 expression attest to the fact that these cells are most likely making very low levels of IL-2 and are therefore not highly activated T cells. Importantly, IL-2 has also been shown to promote the expression of the IL-7Rα chain, which is needed to maintain memory T cells (223). Expression of this receptor was the highest on T cells expanded in alternating gamma chain cytokines, indicating these cells may be able to respond better to homeostatic cytokines once in vivo and that just a one-time pulse of IL-2 was in fact sufficient to prevent receptor downregulation by IL-7. It is noteworthy that although IL-2 increases IL-7Rα expression while IL-7 causes its downregulation (254), T cells expanded in alternating cytokines had the highest expression of the IL-7Rα chain, exceeding levels seen in IL-2.
culture alone, despite the fact that staining was done 4 days after the removal of IL-2 from culture. When combined with the fact that CD25 expression on CD8 T cells expanded in alternating cytokines are at an intermediate level, thus reducing activation induced cell death, we can conclude that T cell expansion in alternating gamma chain cytokines provides a superior method by which to generate highly viable, cytotoxic T cells *in vitro*. In addition to T cell phenotype, function, and viability, we wanted to minimize the expansion of regulatory T cells during the *in vitro* expansion of neu specific activated T cells. Staining for CD4+CD25+Foxp3+ cells was done after expansion in each of the three cytokine regimens. Expansion with IL-7/15 generated the lowest percentage of CD4+CD25+Foxp3+ cells and also resulted in the lowest absolute numbers of cells on day 7. Alternatively, while expansion with alternating cytokines generated the highest percentage of CD4+CD25+Foxp3+ cells, this method also resulted in the greatest fold expansion *in vitro*. In particular, the development of regulatory T cells has been of concern when using IL-2 for T cell expansion, as IL-2 has been shown to be essential for regulatory T cell development (142). However, although we saw that 10% of CD4+CD25+ cells were also positive for Foxp3 expression after IL-2 expansion, these cells exhibited an almost 10-fold expansion, whereas IL-7/15 expanded cells caused only 4% of CD4+CD25+ cells to express Foxp3 and expanded only about 6-fold. Therefore, the generation of cells with a regulatory T cell phenotype by IL-2 in *in vitro* expansions does not seem to suppress cytokine-driven T cell proliferation.

**AIT Using T cells Expanded in Alternating Gamma Chain Cytokines is only Effective when Combined with MDSC Depletion**
Despite the advantages of T cell expansion in alternating gamma chain cytokines, adoptive transfer of these T cells did not overcome the pre-existing immune suppressive microenvironment in FVBN202 animals. We have previously shown that these mice have increased levels of MDSC, even at the pre-malignant stage of mammary hyperplasia (172, 221). However, the combination of depleting MDSC in vivo with AIT expanded in alternating cytokines resulted in a robust tumor regression as seen previously with IL-2 expanded T cells and MDSC depletion and the generation of an antibody response against neu. Complete rejection of MMC tumors by the effector T cells, however, was hindered by the fact that antibody-mediated MDSC depletion in FVBN202 mice was incomplete. This resulted from increasing numbers of MDSC in tumor-bearing mice over time following the cessation of injection of anti-Gr1 antibody, as preliminary depletions (9 days after tumor challenge) were nearly 100% effective. However, by day 35 these levels had increased dramatically and we were not able to inject the rat anti-mouse Gr1 antibody more than 6 times because of its toxicity.

It is important to note that when comparing the distributions of T cell phenotypes and the absolute numbers of T cells from the spleens of mice that received either AIT alone or AIT with MDSC depletion, there were no substantial differences. Mice receiving AIT with MDSC depletion did not retain a greater number of either CD4 or CD8 T cells than mice that received only AIT or MMC. Furthermore, MDSC depletion in mice receiving AIT did not aid in maintaining higher proportions of activated, memory, or effector T cells in comparison to mice that received AIT alone. Therefore, it can be concluded that tumor rejection resulted from a lack of MDSC during the effector phase of the injected T cells.
prior to contraction and not from any subsequent alteration in T cell phenotype, number, or
distribution pattern. To confirm that the type of T cells (effector vs. central memory) and
migratory patterns were not the main determinant of the efficacy of AIT, we injected mice
with either CD62L+ cells (mostly effector memory and a small number of naïve) that
would home to the lymph nodes, or CD62L- cells (mostly effector cells) that could go
directly to the tumor site, after expansion in alternating cytokines. We found that AIT with
either population, in the absence of MDSC depletion, resulted in tumor growth rates
extremely similar to control mice receiving MMC alone. Furthermore, neither group
generated an antibody response, confirming that MDSC depletion alone, and not the state
of T cell differentiation or homing pattern, is responsible for the generation of antibodies
and tumor rejection.

The fact that these experiments use expanded T cells that recognize neu as foreign,
yet still fail to reject MMC tumors by themselves, attests to the strength of in vivo
suppression by MDSC, and upon proving that MDSC depletion does indeed facilitate an
otherwise ineffective AIT, we would next hope to use these procedures with the expansion
of splenocytes from tumor sensitized FVBN202 mice. In order to mimic the high affinity
response seen from FVB lymphocytes, FVBN202 lymphocytes can be expanded while
excluding those T cells having a lower affinity for the neu antigen. MHC-tetramer staining
complexes loaded with a cocktail of the most immunodominant epitopes of neu, and
fluorescently labeled, could be used to sort high affinity FVBN202 T cells at the start of
the procedure. The allogeneic model of rat neu expression in FVBN202 mice is a relevant
model in which to investigate breast cancer immunotherapy because, like human tumors,
the oncogenic protein is largely tolerated. The presence of this rat protein could mimic the fact that human tumors frequently have mutated oncogenes, which are no longer completely tolerated as a normal syngeneic protein would be.

Although other groups have reported the role of MDSC in suppression of anti-tumor T cell responses (166, 167, 255), we have found that MDSC also suppress humoral immune responses following AIT so that depletion of MDSC in vivo restored anti-neu antibody responses in FVBN202 mice. This is very important because it has been reported that collaboration of humoral and cellular immune responses is required for optimal elimination of HER2/neu positive tumors (228). In addition, a novel HER2/neu specific antibody, Pertuzumab, is currently in phase III trials and has been shown to have anti-tumor function through recognition and blockade of the dimerization domain of HER2/neu, ECDII (256). Curiously, there was no antibody response in the groups that received AIT alone, indicating that the presence of adoptively transferred CD4+ T cells alone is not sufficient to facilitate IgG1 isotype switching by the recipients’ B cells because of the presence of MDSC. It is unclear at this point whether the restoration of the antibody response results from lifting MDSC suppression of B cells as well. The role of MDSC in the suppression of the humoral response should be further investigated and could have many applications beyond cancer immunotherapy, since increased MDSC have also been seen in some parasitic infections such as Trypanosoma cruzi (257) and in cases of polymicrobial sepsis (258).
AIT in Conjunction with Gemcitabine Results in Complete Tumor Rejection and Long-Lasting Immunological Memory

In light of these findings, we thought it extremely important to develop a regimen of AIT with MDSC inhibition or depletion that could be more clinically relevant. Previous studies have shown that the use of Gemcitabine can eliminate CD11b+Gr1+ cells in Balb/c mice with neu-expressing tumors, and shows promise when used in conjunction with vaccination strategies (259). We therefore decided to test Gemcitabine in conjunction with AIT for the treatment of MMC tumors. Gemcitabine did in fact significantly reduce MDSC in tumor-bearing FVBN202 mice, and by itself caused a significant inhibition of tumor growth. However, the combination of Gemcitabine with AIT resulted in 100% complete tumor rejection in FVBN202 mice. The effect of Gemcitabine in inhibiting MDSC has recently been shown to be independent of its direct anti-tumor effects, as a single dose of Gemcitabine to mice with established 4T1 mammary carcinomas was able to significantly reduce the MDSC burden in as little as 24-48 hours, prior to any noticeable reduction in tumor volume (260). It is also important to note that lymphocytes isolated 24-48 hours after a single dose of Gemcitabine exhibited increased IFN-γ responses towards 4T1 cells and increased expansion in vitro as compared to lymphocytes isolated from the spleens of control mice (260), thus confirming the functional significance of specific MDSC inhibition by Gemcitabine treatment. The modest tumor inhibition seen in mice receiving Gemcitabine alone or AIT alone in these studies confirms the previous finding that both effector T cells and MDSC inhibition are needed to facilitate tumor rejection. Importantly, mice treated with Gemcitabine and AIT also maintained a healthy weight and appearance.
and exhibited strong antibody responses against ECDII of neu, which was not the case in mice treated with Gemcitabine alone. The generation of an antibody response specifically against ECDII of neu is significant in the fact that we have shown that in FVB mice, responses against this domain are protective and sufficient for tumor rejection, while responses against ECDIV are not protective. Antibody responses against ECDII of neu are of great importance since this could inhibit the dimerization of neu with all other ErbB molecules.

Since one important goal of AIT is to generate immunological memory, we decided to re-challenge all 5 mice that had previously received AIT and Gemcitabine and rejected MMC tumors. Re-challenge was done 74 days after the initial challenge to test for long-lasting immunity. All mice received only 2 doses of Gemcitabine, as the recall response was powerful enough to eliminate the need for the third dose of Gemcitabine. None of the 5 mice grew tumors large enough to be accurately measured by a digital caliper, and all 5 mice quickly became tumor free. Serum taken 12 days after this re-challenge showed very high antibody titers in 2 mice, slightly elevated titers in 2 mice, and a low titer in one mouse. It is important to note that several of the titers reached levels much higher than those observed after the first challenge, consistent with the increased strength characteristic of a recall response. IFN-γ responses to MMC from 2 of the mice confirmed the strong recall response against MMC and indicated that tumor rejection was most likely immune mediated and not mediated by Gemcitabine. Therefore, 18 days after the first re-challenge, the 3 remaining mice were challenged again with MMC but were not treated with Gemcitabine. Two of the mice never developed measurable tumors, while the third
developed a small tumor that was subsequently rejected. Serum taken 16 days after this second re-challenge indicated that antibody titers were again elevated against sudydomain II of neu. The fact that no Gemcitabine was given after this re-challenge suggests that the existing high antibody titers in these mice against ECDII was able to mediate tumor rejection without allowing tumors to reach a size that would cause an increase in MDSC. It is interesting to note that the mouse that developed a small tumor after the second re-challenge was the mouse with the lowest antibody titer out of the three on day 16. Therefore, while we have shown that Gemcitabine does reduce MDSC, treatment with Gemcitabine alone is not sufficient for the rejection of MMC tumors. However, the combination of Gemcitabine with AIT results in complete tumor rejection, the generation of antibody responses, and long-lasting humoral and cellular immunity capable of rejecting further challenge without the anti-tumor effects of Gemcitabine.

Ongoing studies include long-term follow-up of mice treated with AIT and Gemcitabine in order to determine if these mice develop relapsed tumors that may lack expression of neu, as has been observed in one mouse thus far. We have previously shown that IFN-γ can cause downregulation of neu expression on MMC cells in vitro and the emergence of ANV cells in vivo after initial immune-mediated rejection of MMC cells in FVB mice (136). Therefore, it is important to determine if the anti-tumor response that was able to reject 3 MMC challenges in FVB N202 mice given AIT and Gemcitabine may lead to tumor relapse through the process of tumor immunoediting (136). The ANV phenotype of one relapsed tumor (Fig. 33) indicates, along with the in vitro IFN-γ responses observed (Fig. 31) indicates, preliminarily, that strong IFN-γ responses may lead to immune-mediated
tumor escape variants with a more aggressive phenotype. Also of importance is to
determine if T cell-mediated rejection of MMC challenge can be achieved when T cells are
isolated from tolerant FVBN202 mice, expanded in alternating gamma chain cytokines,
and used in conjunction with Gemcitabine. The improved expansion method may be
capable of generating highly effective anti-tumor T cells even from FVBN202 mice and
the ability of Gemcitabine to directly inhibit MDSC could eliminate any tolerance that may
be exerted on these cells. In the clinical setting, where T cells would be taken from a
tumor-bearing individual, it may be important to pre-treat the individual with Gemcitabine,
as has been done by Le et al in the 4T1 mouse model of mammary carcinoma. Pre-
treatment of tumor-bearing mice with Gemcitabine resulted in significantly higher levels of
lymphocyte expansion and increased subsequent IFN-γ secretion after sacrifice (260).
However, these expanded cells were not able to mediate tumor regression when transferred
into mice with established 4T1 tumors (260). In this experiment, the recipient mice were
not treated with Gemcitabine, and, given their established tumors, likely had elevated
levels of MDSC which inhibited the transferred T cells. Future studies should therefore use
Gemcitabine to pre-treat the donor prior to the isolation of cells for expansion, and should
subsequently treat the recipient with Gemcitabine to allow the transferred T cells to be
effective.
II. Chapter 2

Suppression by MDSC is Contact-Dependent

In order to further confirm that failure of AIT to induce regression of MMC in FVBN202 mice was indeed a result of inhibition of T cell function by MDSC, we performed in vitro assays to assess MDSC-mediated inhibition of T cell proliferation. Consistent with the fact that tumor-bearing animals exhibit about a 4-fold increase of MDSC in the granulocyte region of their splenocytes (172), total splenocytes from tumor-bearing mice showed a marked reduction in the number and percentage of proliferating T cells. Significantly, depleting the MDSC from the culture using either an anti-CD11b or an anti-Gr1 antibody caused the restoration of TCR-mediated T cell proliferation. These observations suggest that MDSC suppress TCR-induced proliferation of T cells. To further determine exactly which MDSC were suppressive against CD3/CD28 stimulation, CD11b+ cells were isolated from tumor bearing mice and sorted based on the differential expression of the Gr1 epitopes, Ly6G and Ly6C. Sorting based on these parameters resulted in 2 populations, one that was Ly6G+Ly6C+ and one that was Ly6G-Ly6C+. Suppression assays using these cells instead of whole CD11b+Gr1+ MDSC showed a significant reduction in CD3/CD28 mediated T cell proliferation only in the presence of Ly6G-Ly6C+ cells. This is in contrast to recent reports that have shown different subsets of MDSC to be equally suppressive. Specifically, reports by Movahedi et al using MDSC from EG7 thymomas and BW-Sp3 lymphomas and Youn et al, using MDSC from EL-4 thymomas have shown that both subsets of MDSC are suppressive against OT-1 splenocytes stimulated with specific peptide; however, neither group reported suppression
against splenocytes stimulated with anti-CD3/CD28(171, 215). Our subsets reported here, therefore, may represent unique populations of MDSC found in MMC tumor-bearing FVBN202 mice. Consequently, in this model, broadly defining MDSC as CD11b+Gr1+ cells is inaccurate as not all of these cells are suppressive. The term MDSC in regards to the FVBN202 mouse model should therefore be defined specifically as CD11b+Ly6G-Ly6C+ cells.

The possible contact-dependent mechanism of T cell suppression by MDSC has been an area of some debate. Using different tumor models, most groups have found suppression of T cells by MDSC to be mediated by soluble factors such as arginase-1, nitric oxide, reactive oxygen species and peroxynitrites (173, 261). In particular, arginase-1 has been implicated in downregulation of the TCR zeta chain and the induction of this enzyme has been linked to tumor-derived soluble factors such as prostaglandin E2 as well as IL-4 and IL-13 (173, 182, 188). Furthermore, arginine-depleted conditions can cause TCR zeta downregulation in the absence of cell-to-cell contact (188). Indoleamine 2,3-dioxygenase, has also been found to be expressed in macrophages and dendritic cells as well as human tumors and contributes to T cell anergy CD8 T cell suppression through the catabolism of tryptophan(186, 187, 262). Inconsistent expression of IL-10 and extremely low expression of IDO lead us to conclude that these molecules were not paramount in mediating suppression by MDSC. Furthermore, a role for TGF-β was excluded as higher expression of this molecule was seen in the non-suppressive population of CD11b+Ly6G+Ly6C+ cells than was observed in the CD11b+Ly6G-Ly6C+ suppressive population. This led us to hypothesize that suppression by MDSC in our model may be
contact-dependent. Indeed, Transwell assays confirmed that cell-to-cell contact was required for the suppression of T cell proliferation. Although other mechanisms may be involved in suppression of cytotoxic responses by T cells, we report here that no suppression of T cell proliferation was seen when MDSC were added to the top chamber of a transwell insert. Nagaraj et al have recently shown that direct cell-to-cell contact between CD8+ T cells and MDSC causes nitration of tyrosines in the TCR-CD8 complex, therefore disrupting binding of specific antigen-MHC class I complexes to the TCR’s of OT-1 transgenic T cells (183). It is unclear, however, if the effects of this nitration may be exaggerated by transgenic expression of the TCR and what role this mechanism may play in a TCR non-transgenic model. Furthermore, Gabrilovich et al have reported that blocking the MHC class I molecules expressed on the surface of MDSC can reverse suppression of CD8+ T cells, which involved MDSC production of nitric oxide, but reported that these cells did not suppress CD4+ T cell responses towards MHC class II presented peptides (166). In 2000, Kusmartsev et al. showed inhibition of CD3/CD28 T cell activation by MDSC isolated from mice bearing MCA-26 colon carcinomas. However, this suppression was reversed by the addition of a superoxide dismutase mimetic and a nitric oxide synthase inhibitor and a possible role of contact was not investigated, and proliferation was not determined in separate populations of CD4+ and CD8+ T cells (170). In contrast, we show here that MDSC from mice bearing neu+ mammary carcinomas inhibit the proliferation of both CD4+ and CD8+ T cells in contact-dependent manner. Therefore, although contact between MDSC and CD8+ T cells has been speculated to be important in inhibiting the IFN-γ response of CD8+ T cells towards specific peptide, we show here that contact is also
necessary to inhibit CD3/CD28 T cell stimulation and can affect the proliferation of both the CD4+ and CD8+ T cells populations.

**Contact-Dependent Suppression is Independent of CD86, LFA-1, and PD-L1**

The inhibitory receptor CTLA-4 (CD152) is rapidly upregulated upon T cell activation and has a higher affinity for the co-stimulatory molecule CD86 (B7-2) than its stimulatory counterpart, CD28 (263, 264). Furthermore, CD86, while expressed at low levels on resting APC, is also rapidly upregulated following activation (265). We therefore hypothesized that increased expression of CD86 on activated MDSC from tumor-bearing mice may cause the inhibition of T cells through CTLA-4. While this mechanism is normally in place as a regulator of the immune response, the increased frequency of MDSC present in tumor-bearing individuals could potentially cause an overwhelming amount of negative signaling through CTLA-4 and negate any and all positive signals though CD28. However, we were surprised to see that the percentage of CD11b+Gr1+ cells from tumor-bearing mice expressing CD86 was actually significantly lower than CD86 expression on CD11b+Gr1+ cells from tumor free mice. Although it is therefore unlikely the CD86 is a mediator of active suppression by MDSC, considering that MDSC make up over 90% of the granulocyte region in tumor-bearing mice (see Figure 5) and only about 30% of these MDSC express CD86, it is likely that contact between TCR-MHC of T cells and MDSC results in T cell anergy, which results from TCR activation in the absence of co-stimulation (266).
In addition to CD86, we were also able to rule out MDSC-mediated interactions through LFA-1, which is also highly expressed by CD11b-Gr1- cells and therefore would not limit suppression to the CD11b+Gr1+ population. Contact of the PD-1 molecule on T cells by PD-L1 on MDSC’s seemed likely as PD-L1 is known to be expressed on dendritic cells and macrophages and can inhibit TCR-induced proliferation, induce T cell anergy, or cause apoptosis (241-243). However, we saw no expression of PD-L1 on CD11b+Gr1+ cells, and therefore are able to rule out contact through the PD-1/PD-L1 axis as a means of contact-dependent suppression. We can therefore conclude that tumor-bearing FVBN202 mice have unique subsets of MDSC, only one of which is suppressive, and that this suppression requires direct contact with T cells that is independent of interactions with LFA-1, PD-L1, and CD86. Future studies aim to identify the exact point or points of contact required between T cells and MDSC for MDSC to mediate T cell suppression.

III. Chapter 3

MMC-Derived Soluble Factors Drive the Accumulation of MDSC

The accumulation of MDSC in tumor-bearing hosts is a major obstacle to the effective treatment of cancer (267) and many studies have shown a link between tumor-derived soluble factors and increased MDSC. Identification of such soluble factors could offer therapeutic intervention to avoid generation of MDSC. Injection of MMC conditioned medium confirmed that MMC-derived soluble factors did indeed cause a significant increase in the proportion of the granulocytes that were MDSC, as well as causing a significant difference in the overall percentage and absolute numbers of MDSC
in the spleen, blood, and bone marrow of the tibias and femurs. The fact that MMC-derived soluble factors also caused a significant decrease in the percentage of MDSC expressing the MHC class II molecule I-A<sup>d</sup> lead us to hypothesize that in addition to increasing MDSC, tumor-derived soluble factors may also play a role in dampening the immune response by reducing antigen presentation. In support of this hypothesis, we did in fact find expression of I-A<sup>d</sup> to be significantly decreased in the CD11b+Gr1- DC population in mice receiving injection of MMC conditioned medium as compared to mice receiving control medium. Since mature APC’s are one of only a few cell types that constitutively express MHC class II molecules, and since antigen stimulated helper CD4+ T cells are imperative for somatic hypermutation and isotype switching of B cells as well as for the induction of cytotoxic CD8+ T cell responses, the decrease in APC’s expressing MHC class II could negatively affect the ability of the host to respond to tumor antigens and mount an effective anti-tumor response (268). Interestingly, there was not a measurable difference in the expression of the MHC class I molecule, H-2<sup>d</sup> on CD11b+Gr1- cells, indicating that inhibition of the anti-tumor response occurs mainly through the inhibition of helper T cells. Our studies also found that MMC derived supernatant did not influence the expression of AnnexinV, CD86, CD11c, H-2<sup>d</sup>, Qa1, or CCR7 on CD11b+Gr1+ cells.

A multiplex cytokine array to determine the main soluble factors secreted by MMC returned interesting results. We found that MMC cells secrete large amounts of VEGF and MCP-1 and IL-6, as well as smaller amounts of GM-CSF. It is also important to note that these cells do not produce substantial mIL-1β, which has been shown by Bunt et al to
increase MDSC’s in a 4T1 model (269). Also they do not secrete measurable IL-4 or IL-13; it has been published that MDSC’s can respond to IL-4 and IL-13 in a way that causes them to take on an alternatively activated phenotype (167). The lack of secretion of IL-10, which is important in inducing the suppressive phenotypes in Tregs, indicates that these cells probably do not play a crucial role in this model (270).

Despite secretion of GM-CSF, VEGF, IL-6, and MCP-1 by MMC cells, injection of these cytokines in vivo did not result in statistically significant increases in MDSC in the spleen, bone marrow, and blood of FVBN202 mice, as was seen after injection of MMC-conditioned media. We believe this is because of the short half–lives of these molecules, as groups that have seen in vivo responses have used a means of continuous in vivo administration such as the use of GM-CSF-transfected melanoma cells that secrete up to 6000ng per 1X10^6 cells per hour, or subcutaneously implanted osmotic pumps delivering VEGF at a constant rate of 50 or 100ng per hour, which has been shown to have a half life of only 25 minutes (208, 271, 272). We therefore decided to use in vitro studies to analyze the effects of these cytokines/chemokines on sorted populations of both CD11b-Gr1-progenitor cells and CD11b+Gr1+ MDSC from the bone marrow of FVBN202 mice.

**GM-CSF is the Main Determinant of MDSC Generation and Survival**

GM-CSF has been used in dendritic cell vaccination strategies to induce tumor-specific immune responses. For example, Driessens et al have recently found that administration of GM-CSF-transduced renal cell carcinoma cells along with dendritic cells is able to mediate responses in 50% of mice and also resulted in immunological memory (273). GM-CSF is a crucial factor in the generation of dendritic cells and plays an
important role in regulating and activating these cells and has been shown to increase numbers of circulating dendritic cells in patients receiving exogenous GM-CSF with along with either G-CSF or Flt3 (274, 275). Patients who received GM-CSF with Flt3 generated increased numbers of DC1 cells, which stimulate a Th1 response, while patients who received GM-CSF in conjunction with G-CSF showed increased DC2 cells, which cause a Th2 polarization (275). This is not surprising given fact that GM-CSF has a broad range of biological activity and can exert different effects in varying cells types depending on concentration, differentiation status, and surrounding environment and cytokine milieu (276). Showing the diversity of responses elicited by GM-CSF, a recent study in patients with stage IV melanoma has shown that administration of GM-CSF in combination with a multi-epitope vaccine offered no improvement in anti-tumor immune responses as compared to those seen in patients that received vaccination alone (277), while another has shown that vaccination of patients with metastatic melanoma using irradiated autologous melanoma cells engineered to express GM-CSF resulted in anti-tumor responses in 11 out of 16 patients (278).

Given that the role of GM-CSF on MDSC has only recently been investigated and considering the heterogeneity of responses that can occur as a result of GM-CSF, we found it imperative to investigate the role of this cytokine in MDSC generated in the FVBN202 mouse model. In agreement with literature that has implicated GM-CSF as an important factor in the development and differentiation of a variety of hematopoietic cells (276), we found that total bone marrow cells, most of which were CD11b+Gr1+, cultured with GM-CSF resulted in the generation of a large population of CD11b+Gr1- dendritic cells.
Despite the fact that the CD11b+Gr1+ population dropped from 77% of the total bone marrow on day 0 to 25% on day 6, quantification of the absolute number of these cells showed that this population actually increased, albeit not significantly, during this time period. This led us to conclude that GM-CSF is in fact maintaining the MDSC population throughout the culture period. Furthermore, CD86 expression on MDSC was significantly increased by day 6 of culture, which is of interest given the previous finding that CD86 levels on MDSC were significantly reduced on MDSC from tumor-bearing mice as compared to those from tumor-free mice (see Figure 39). Therefore while GM-CSF alone may result in MDSC with a slightly more mature phenotype, the presence of other tumor-derived soluble factors in conjunction with GM-CSF negates this effect.

Although we did not see a significant difference in CD86 expression on MDSC after injection with MMC-derived supernatant, it is possible that a longer time period is required for this effect, as only 3 injections of MMC supernatant over the course of 3 days were given. Contrary to the increase in CD86, 6 days of culture with GM-CSF caused MDSC in the bone marrow to drastically downregulate their expression of the chemokine receptor CCR7. CCR7 has only 2 ligands, CCL19 and CCL21, which are important in guiding CCR7-expressing cells through high endothelial venules and into the lymph nodes (279). CCR7 is expressed only on semi-mature and mature DC’s, and like CD86, is greatly upregulated upon DC maturation. Although DC’s in Ccr7−/− mice do not migrate to the draining lymph nodes after activation, T cells lacking CCR7 are still found in the red pulp of the spleen and B cells were found in both the red and white pulp of the spleen, indicating that migration into the spleen is still possible in the absence of CCR7 (246).
Furthermore, it has been found that the addition of CCL19 to DC cultures induced their maturation and that CCR7 ligands can increase the ability of mature DC’s to take up antigen (280, 281). In light of these findings, although the upregulation of CD86 on MDSC may indicate a maturing effect of GM-CSF on these cells, the concurrent downregulation of CCR7 may prevent complete maturation, antigen uptake, and migration to the lymph nodes while explaining the preponderance MDSC present in the spleens and periphery of tumor-bearing mice, while increases have not been observed in the lymph nodes (unpublished observation). We next sought to determine the effects of GM-CSF specifically on existing MDSC or on the generation of MDSC by using pure sorted cell populations.

The important role of GM-CSF in the expansion of MDSC has been suggested previously by other studies. For example, GM-CSF-secreting tumors and GM-CSF administration \textit{in vivo} (5\mu g twice daily for 3 days) have been implicated in increased numbers of MDSC (282). It was determined that only high-dose GM-CSF had such deleterious effects (271), though differential effects of GM-CSF on progenitor cells versus existing MDSC were not determined. We have determined that GM-CSF is the major tumor-derived soluble factor that causes both the generation of MDSC from sorted CD11b-Gr1- cells and maintains existing sorted CD11b+Gr1+ MDSC for the duration of a 6 day culture. Interestingly, CD11b+Gr1- cells were not abundant after culture of either of the sorted fractions with GM-CSF, indicating that these cells may have, in part, expanded from the existing 4% present on day 0 (see Figure 58). However, there was a substantial difference on day 6 between cells cultured in GM-CSF as compared to cells cultured with
MMC in Transwell. While the presence of GM-CSF alone with sorted CD11b-Gr1- cells drove the production of a majority of MDSC, the presence of MMC with CD11b-Gr1- cells resulted in an almost equal distribution of CD11b+Gr1+ cells and CD11b+Gr1- cells on day 6. This indicates that tumor-derived soluble factors other than GM-CSF are responsible for the generation of CD11b+Gr1- cells and explains a previous observation that the tumor site contains a higher proportion of CD11b+Gr1- cells than the spleen, bone marrow, or blood of FVBN202 mice (see Figure 5). In contrast, the effect on sorted CD11b+Gr1+ cells was reversed, with culture with GM-CSF alone resulting in a greater percentage of CD11b+Gr1- cells (21.8%) than culture with MMC in Transwell (only 7.3%). This shows that CD11b+Gr1+ cells are in fact capable of converting into CD11b+Gr1- cells, but that the presence of other tumor-derived soluble factors besides GM-CSF largely prevents this from occurring. Secondly, this highlights the drastically different effects that the same treatment (GM-CSF or MMC cells) can have on these two different cell populations. In fact, our data indicated that sorted CD11b-Gr1- progenitor cells were more susceptible to the effects of GM-CSF not only in the generation of MDSC, but also for the survival of these MDSC as compared to CD11b+Gr1+ bone marrow cells. Although GM-CSF, and to a lesser extent MMC-derived soluble factors, significantly augmented the viability of both existing and newly generated MDSC, lower AnnexinV staining in newly generated MDSC derived from CD11b-Gr1- cells may be a result of high turnover rates in MDSC such that previously generated MDSC are more prone to apoptosis than newly generated MDSC even in the presence of GM-CSF. Interestingly, in both cases, the protective effects of GM-CSF on MDSC appeared to be dose-dependent, because
culture with GM-CSF at 100ng/mL caused a significant reduction in apoptosis as compared to MMC secreting ~350 pg/mL GM-CSF.

**MMC-Derived Soluble Factors or GM-CSF Support the Generation of a Suppressive Ly6G-Ly6C+ MDSC Subset.**

Identifying MDSC subsets is an important factor that may lead to more efficient therapeutic means of eliminating or inhibiting these specific cell populations, while minimizing undesirable side effects. We therefore sought to further characterize MDSC subsets in FVBN202 mice through differential expression of the Gr1 epitopes Ly6G and Ly6C. It has recently been reported that expression of Ly6G correlates with a granulocytic phenotype, while expression of Ly6C correlates with a monocytic phenotype (171, 215). The tumor-bearing FVBN202 transgenic mouse model of neu positive mammary carcinoma displays novel phenotypes and characteristics of MDSC. Although Youn et al observed increases in MDSC subsets in FVBN202 mice with ANV tumors, FVBN202 bearing MMC tumors were not evaluated (171). Two distinct subsets exist in MMC tumor-bearing FVBN202 mice, Ly6G-Ly6C^{low/int}, and Ly6G+Ly6C^{high}. Previously reported subsets include Ly6G-Ly6C^{high}, Ly6G^{+}/Ly6C^{low/int}, and Ly6G^{int/dull}Ly6C^{hi} (171, 215, 216). These phenotypes display an inverse correlation between Ly6G and Ly6C expression, where high Ly6G expression is associated with intermediate or low Ly6C expression and low or absent Ly6G expression is associated with high Ly6C expression, which is in direct contrast to the phenotypes presented here.

In addition to previously reported differences in suppressive activity of these two subsets (see Figure 35), the expansion of MDSC subpopulations has also differed among
groups. Like Youn et al, we observed the greatest expansion in the Ly6G positive subset in the spleens of tumor-bearing mice as compared to tumor-free mice (Figure 62), however we observed similar expansion of the two subsets in the bone marrow (171). In contrast, Movahedi et al have reported a roughly equal distribution of Ly6G+ and Ly6G- subsets in tumor-bearing spleens, and have also found both populations to expand with similar kinetics (171, 215).

We next sought to determine the effects that GM-CSF or MMC would have on the distribution or expansion of these MDSC subsets. Strikingly, culture with 100ng/mL of GM-CSF, and to a lesser extent MMC, also resulted in a significantly higher percentage of Ly6G- cells, previously found to be suppressive, and a significantly lower percentage of Ly6G+ cells, which have shown no suppressive activity in this model. Like the protective effects of GM-CSF, the reversal of the distribution of these cell populations seems to be concentration dependent, as the Ly6G- population was also significantly increased as compared to that seen after culture with MMC. It is interesting to note that the MFI if Ly6C was greatly increased after culture with GM-CSF or MMC as compared to that seen in the bone marrow on day 0. Considering there is a slight increase in the MFI of Ly6C in the bone marrow of tumor-bearing versus tumor free mice, high expression of Ly6C may represent a marker of activated MDSC with increased suppressive activity. The suppressive capacity of Ly6G-Ly6C\textsuperscript{high} cells remains to be determined and should be compared to the suppressive effect seen previously in the Ly6G-Ly6C\textsuperscript{low/int} population. Additionally, variances in the amount of GM-CSF secreted by other tumor cell lines may explain the different subsets that have been found in different models.
Further characterization of the effects of GM-CSF and MMC-derived soluble factors on sorted CD11b-Gr1- progenitor cells revealed that, consistent with previous findings, both the MHC class I (H-2^d) and the MHC class II (I-A^d) molecules were significantly reduced on MDSC that had arisen from these cells after 6 days. This suggests that any cells arising under conditions of elevated GM-CSF may have stunted antigen presentation capabilities. We did not, however, observe significant differences in H-2^d and I-A^d levels after the culture of sorted CD11b+Gr1+ cells, again highlighting the increased susceptibility of progenitor cells to the effects of GM-CSF. This also explains the more pronounced difference seen in the expression of these molecules as compared to that seen previously after injection of MMC-conditioned medium, since these populations would include cells that were already CD11b+Gr1+ as well as cells that were CD11b-Gr1- at the time of injection (see Figures 46 and 47). Therefore, it has been consistently found that the presence of GM-CSF, either alone or as secreted by MMC, can reduce the antigen presentation abilities of both CD11b+Gr1+ as well as CD11b+Gr1- cells and may play a role in preventing the full maturation of these cells, which is characterized by increases in MHC class II expression, upon antigen uptake (279). GM-CSF does not, however, cause the proliferation of existing sorted MDSC which is consistent with the fact that we did not see a statistically significant difference between the absolute number of MDSC recovered from the bone marrow of the tibias and femurs on day 0 as compared to after 6 days of culture with GM-CSF. This is likely a result of the rapid turnover rate of MDSC in which existing MDSC are more prone to apoptosis and will be replaced by newly arising MDSC from the bone marrow. It is likely, however, that the accumulation of increasing numbers
of MDSC is a result of the generation of new MDSC outpacing the apoptosis of existing MDSC, and this may also account for the non-significant increase in MDSC numbers seen in Figure 58 as GM-CSF drove the production of CD11b+Gr1+ cells from CD11b-Gr1- bone marrow progenitor cells.

Given the effects of GM-CSF on CD11b+Gr1+ and CD11b+Gr1- cells, it is conceivable that, since GM-CSF has been found to be secreted in 31% of tested human tumor cell lines (282), administering GM-CSF in the clinical setting may have an additive effect with tumor-secreted GM-CSF and therefore support the generation of MDSC from bone marrow progenitor cells, as well as maintaining existing populations of MDSC and driving the production of highly suppressive subsets of MDSC. A recent review on the opposing effects of GM-CSF as an adjuvant in cancer patients also suggested activation and expansion of MDSC by endogenous tumor-derived or exogenous GM-CSF (211). However, a study on patients with high-risk melanoma treated with adjuvant GM-CSF showed that GM-CSF mainly expanded DCs rather than MDSC (283). The authors used CD33+HLA-DR- as markers for MDSC whereas, using CD34+ as a marker for MDSC, others found that patients with head and neck squamous cell carcinoma (HNSCC) had profound immune deficiencies associated with an increased intra-tumoral presence of immune-suppressive CD34+ progenitor cells (210). Therefore different observations may be attributed to different markers used for identification of human MDSC. Although markers of MDSC in humans are not as well characterized as in mouse, we made similar observations in mice when using unfractionated bone marrow cells in culture with GM-CSF. This suggests a dual role for GM-CSF in the generation of DCs and MDSC. The
frequency of distinct progenitor cells for DCs or MDSC in the bone marrow of cancer patients may determine which effect of GM-CSF may prevail. Given these findings, existing serum levels of MDSC in patients should be considered before administering GM-CSF as an adjuvant therapy and levels should be monitored closely during treatment in an effort to minimize the deleterious effects of GM-CSF.

Interestingly, we observed a complete lack of response when using either VEGF or MCP-1 in culture with sorted CD11b-Gr1- cells or CD11b+Gr1+ cells. This was somewhat surprising given that MMC cells secrete over 4.5 times as much VEGF and over 3 times as much MCP-1 as GM-CSF. In particular, recent evidence has pointed towards VEGF as an important mediator of myeloid cell accumulation and differentiation through the activation of STAT3 which has been shown to prevent the differentiation of myeloid cells (201). Increased levels of VEGF have also been shown to result in the inhibition of DC development and the accumulation of Gr1+ cells (208, 272). Likewise, the link between the chemokine MCP-1 (CCL2) and elevated levels of detrimental tumor-associated macrophages in breast cancer patients made it a likely candidate for causing the generation of suppressive macrophage and/or MDSC populations (284, 285). We have indeed observed a higher percentage of CD11b+Gr1- cells at the tumor site, an effect that is recapitulated in Figure 60B with sorted CD11b-Gr1- progenitor cells cultured with MMC in transwell converting to 43.8% CD11b+Gr1- cells by day 6. However, neither sorted populations of progenitor cells nor MDSC responded to either VEGF or MCP-1 alone. GM-CSF, secreted at an average of 336pg/mL by MMC cells, is therefore the main determinant of MDSC generation and maintenance.
We showed that MMC or GM-CSF specifically supported the generation of the suppressive Ly6G-Ly6C+ subset \textit{in vitro}. On the other hand, tumor-bearing mice showed a higher percentage of Ly6G+Ly6C+ than Ly6G-Ly6C+. Such discrepancies between \textit{in vitro} and \textit{in vivo} observations could be attributed to the higher dose of GM-CSF or MMC in co-culture with the progenitor cells compared to that \textit{in vivo}. Alternatively, immune responses against the tumor may compromise the effects of tumor-derived soluble factors \textit{in vivo} and reduce the proportion of the suppressive subset. This possibility is supported by recent reports showing that immunological tolerance was broken against the neu positive tumors in FVBN202 transgenic mice (221, 286).

GM-CSF therefore is the main factor having the ability to drive the production of new MDSC from bone marrow CD11b-Gr1- progenitor cells, and, more specifically augments the generation of a novel Ly6G-Ly6C+ suppressive subset of MDSC. In contrast, VEGF and MCP-1, both previously linked to MDSC accumulation, had no effect on the generation or maintenance of MDSC. Future studies will determine the suppressive activities of the Ly6G-Ly6C+ and Ly6G+Ly6C+ subsets generated after culture with either GM-CSF or MMC to elucidate if the highly expanded Ly6G-Ly6C+ subset remains suppressive. Furthermore, comparisons between the suppressive activities of CD11b+Ly6G-Ly6C^{low} cells found in naïve FVBN202 mice, the CD11b+Ly6G-Ly6C^{int} cells found in tumor-bearing mice, and the CD11b+Ly6G-Ly6C^{high} cells generated after culture with MMC or GM-CSF should be conducted to determine if expression of the Ly6C molecule may be a prognostic marker for identifying suppressive populations of MDSC.
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296
VITA

Personal Summary:
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Publications:
  o Manjili, M. H., Kmieciak, M., & Keeler, J. (2006). Comment on "tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma". Journal of immunology 176(8), 4511

Poster Presentations:
  o Morales, J.K., Kmieciak, M., Graham, L. Feldmesser, M., Bear, H.D. Manjili, M.H. Adoptive Transfer of HER2/neu Specific T Cells Expanded with Alternating Gamma Chain Cytokines Mediates Tumor Regression when Combined with the


