Novel Role of PPAR-gamma in GM-CSF induced anti-tumor immunity

ABSTRACT

Granulocyte macrophage colony stimulating factor (GM-CSF) mediates context dependent anti- or pro-inflammatory functions through cells of the myeloid lineage. GM-CSF signaling induces the expression of the transcription factor peroxisome proliferator-activated receptor gamma (PPAR-γ). We examined the role PPAR-γ in myeloid cells in the anti-tumor response to GVAX, a GM-CSF based cancer immunotherapy using the B16 model of murine melanoma.

We found that selective loss of PPAR-γ in the myeloid lineage using LysM-Cre reduces the efficacy of GVAX which could not be explained by known mechanisms. RNASeq of GVAX draining lymph node identified an increase in regulatory T-cells markers such as FoxP3 and coinhibitory receptors CTLA-4 and TIGIT in LysM-Cre; PPAR-γ fl mice (PPAR-γ KO). We confirmed by flow cytometry that Treg frequency was indeed increased in PPAR-γ KO lymph node with a strong reduction seen in the ratio of CD8 T-cells to regulatory T cell (CD8:Treg). Treg recruiting chemokines CCL17 and CCL22 were upregulated in the draining lymph node. Importantly, tumors in PPAR-γ KO mice had a reduced CD8:Treg ratio explaining the loss in GVAX efficacy.

Pharmacological activation or inactivation of PPAR-γ in GM-CSF treated human PBMC showed conservation of the role of PPAR-γ in regulating T-cell numbers in humans. PPAR-γ agonism in
mice, using the FDA-approved small molecule ligand rosiglitazone (Rosi), improved CD8:Treg ratios in the vaccine draining lymph node and tumors. The gain-of-function data suggested the Rosi could be used as an adjunct to immunotherapy. All intratumoral Treg expressed high levels of CTLA-4 and TIGIT. Thus, we tested the impact of Rosi on the response to GVAX and anti-CTLA-4 combination therapy. We found that Rosi improved the tumor incidence and overall survival of tumor bearing mice treated with GVAX and anti-CTLA4.

Our data have identified a novel role of PPAR-γ in myeloid cells in regulating Treg numbers. This pathway is conserved in humans as seen in ex-vivo studies of PBMC. Further, we provide preclinical evidence that Rosi can be used to improve immunotherapeutic responses by increasing the ratio between intratumoral effector and regulatory cells.
TABLE OF CONTENTS

i. Title page  
ii. Copyright  
iii. Abstract  
v. Table of Contents  
vi. Dedication  
vii. Acknowledgements  
viii. List of tables and figures  

Chapter 1: Introduction  

Chapter 2: The role of GM-CSF in maintaining PPAR-γ expression in myeloid and non-myeloid populations  

Chapter 3: Effect of genetic loss-of-function in the monocyte lineage on GVAX: studies on candidate mechanisms  

Chapter 4: High throughput analysis of gene expression in GVAX draining lymph node and identification of a novel role of PPAR-γ in myeloid cells  

Chapter 5: Effect of PPAR-γ modulation in studies of GM-CSF function in human PBMC  

Chapter 6: Discussion  

Appendix I: Publications  

1  

31  

42  

65  

87  

94  

106
Dedicated to my father,

S. P. Goyal

1939-1991
ACKNOWLEDGEMENTS

I have been lucky to have the support, friendship and mentorship of many people in the last eight years. I would like to thank my Ph.D advisor Glenn Dranoff, my HGWise mentor Maria Kontaridis, members of my DAC and all my teachers and advisors for their patient support. Several members of the Dranoff lab (past and present) have collaborated with me, challenged my thinking and have become close friends over the years. I would also like to thank my husband Bhavjit and many friends who have enriched my personal and academic life.
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table Number</th>
<th>Title</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Comparison of phenotypes observed in GM-CSF KO and Lys-M-Cre; PPAR-γ fl mice</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>PPAR-γ KO splenocytes restimulated with B16 cells do not show an alteration in their cytokine profile.</td>
<td>56</td>
</tr>
<tr>
<td>3</td>
<td>Chemokines implicated in Treg recruitment</td>
<td>101</td>
</tr>
</tbody>
</table>

LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure Number</th>
<th>Title</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Isoforms and domains of full length PPAR-γ</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>Expression of PPAR-γ in B16 cells and various tissues.</td>
<td>41</td>
</tr>
<tr>
<td>3</td>
<td>Detection of overexpressed and endogenous PPAR-γ protein confirmed a requirement for GM-CSF to maintain PPAR-γ expression in alveolar macrophages.</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>PPAR-γ expression in resting peritoneal macrophages 5 hours after plating.</td>
<td>43</td>
</tr>
<tr>
<td>5</td>
<td>PPAR-γ expression in thioglycollate elicited peritoneal macrophages.</td>
<td>44</td>
</tr>
<tr>
<td>6</td>
<td>PPAR-γ expression in perigonadal adipose tissue.</td>
<td>45</td>
</tr>
<tr>
<td>7</td>
<td>PPAR-γ expression in CD11b depleted splenocytes.</td>
<td>45</td>
</tr>
<tr>
<td>8</td>
<td>Detection of PPAR-γ by flow cytometry.</td>
<td>46</td>
</tr>
<tr>
<td>9</td>
<td>Generation of myeloid specific KO of PPAR-γ</td>
<td>53</td>
</tr>
<tr>
<td>10</td>
<td>Genetic depletion of PPAR-γ in myeloid cells reduces vaccination efficiency in B16 murine melanoma model.</td>
<td>54</td>
</tr>
<tr>
<td>11</td>
<td>CD1d expression remains unchanged in naïve PPAR-γ KO spleen.</td>
<td>57</td>
</tr>
<tr>
<td>12</td>
<td>CD1d expression remains unchanged in vaccinated PPAR-γ KO spleens.</td>
<td>58</td>
</tr>
<tr>
<td>13</td>
<td>Alveolar macrophages from PPAR-γ KO mice retain equivalent surface expression of CD1d.</td>
<td>58</td>
</tr>
<tr>
<td>14</td>
<td>A granulocytic, a monocytic and one DC population can be distinguished at the live-GM vaccine site in equal numbers in con and PPAR-γ KO mice.</td>
<td>60</td>
</tr>
<tr>
<td>15</td>
<td>No difference was detected in activation status of live-GM vaccine site granulocytes, monocytes and DC in PPAR-γ KO.</td>
<td>61</td>
</tr>
<tr>
<td>16</td>
<td>CD1d expression on CD11b SP and CD11b CD11c DP cells recruited to vaccine site was not affected in the PPAR-γ KO mice.</td>
<td>62</td>
</tr>
</tbody>
</table>
PD-L1 expression on myeloid cells recruited to the vaccine site is not affected in the PPAR-γ KO.

Subsets of APC recruited to the vaccine site.

Coculture with naïve or vaccinated CD4 and CD8 live vaccine site APC did not reveal a defect in the PPAR-γ KO.

NKT cells cultured with con or PPAR-γ KO vaccine site APC display similar cytokine profiles.

GSEA shows difference in KO dLN consistent with loss of PPAR-γ in myeloid cells.

GSEA and flow cytometry show increased Treg and decreased CD8:FoxP3 ratio in PPAR-γ KO dLN.

Analysis of tumor infiltrating leukocytes reveals lower T-cell infiltration in tumors in PPAR-γ KO mice.

Analysis of tumor infiltrating leukocytes reveals lower T-cell infiltration in tumors in PPAR-γ KO mice.

KO dLN produce higher levels of Treg attracting chemokines.

Con and KO CD8 from GVAX dLN produce equivalent levels of IFN-γ in response to Trp-2 peptide.

KO LN have increased expression of a Langerhans Cell specific gene module.

LC express modest levels of lysozyme M.

Staining strategy for Langerin expressing DC in the lymph node.

Total CD207+ cells or the frequency of CD103 expression is unaffected in the PPAR-γ KO.

Rosi does not impact the balance between CD8 and Treg in the vaccine draining lymph node after 6-8 days of treatment.

20mg/kg/day Rosi delivered via drinking water improves the intratumoral CD8: Treg ratio in GVAX treated mice.

Rosi mediated improvement in immune correlates requires PPAR-γ expression in myeloid cells.

Rosi potentiates the efficacy of GVAX+CTLA-4 treatment.

Treatment of human PBMC with GM-CSF and PPAR-γ modulators recapitulates Treg effects seen in murine studies.

CCL17 expression by GM-CSF treated human monocytes is reduced upon Rosi treatment.

Analysis of adherent PBMC treated Rosi did not changes in number or activation status.
In this manuscript, we investigate the biological mechanisms underlying the efficacy of the cancer immunotherapy, GVAX. For this immunotherapy, patient tumor cells are harvested and transduced to overexpress a cytokine, granulocyte-macrophage colony stimulating factor (GM-CSF). These cells are then irradiated to prevent tumor growth and used as a subcutaneous inoculum to generate an immune response against the tumor. In the following introduction we first discuss the mechanisms of tumor immunity. Next, we introduce GM-CSF and how it impacts tumor immunity in endogenous and therapeutic settings. Finally, we summarize the literature about GM-CSF target peroxisome proliferator-activated receptor gamma (PPAR-γ) and our rationale to study its role in myeloid cells during an immune response to GVAX.

TUMOR IMMUNITY

The cancer surveillance hypothesis was formalized in 1957 by Burnet and Thomas [1, 2]. It postulates a) the existence of tumor antigens “foreign” to the immune system due to somatic mutations or viral products, b) cancer surveillance by the immune system restricting the growth of the tumor and c) the idea of cancer immunotherapy. The corollary to these postulates (even though no research was conducted on the topic at the time) is that progressive tumors have been immunoedited and have evolved mechanisms for immune evasion.
Early studies

The early search for tumor antigens was confounded by the use of murine xenografts. In actuality, the researchers were describing transplantation antigens. This cast into doubt the existence of tumor antigens in syngeneic tumors. Subsequently, researchers were able to immunize mice with syngeneic tumors such that when challenged with these tumors, the mice showed protection against tumor incidence and growth. These studies showed that tumors could induce an immune response to related tumors and allowed identification of some murine tumor antigens. However, the principle of endogenous anti-tumor immunity was accepted only in the 1990s as a result of the studies described below.

First, T cells generated from patients were shown to be cytotoxic to patient tumor cells [3]. Boon and colleagues used a clonal cell line derived from patient cytotoxic lymphocytes (CTL) to identify the cognate antigen MAGE-1 on melanoma cells. This study was the first to identify a human tumor antigen and proved the existence of endogenous anti-tumor immunity in patients.

Second, the concept of cancer immunosurveillance was demonstrated using mice with reduced interferon-gamma (IFN-γ) function that showed enhanced growth of transplanted tumors [4, 5]. Also, chemically induced tumors in mice with severe combined immunodeficiency (SCID) were more immunogenic compared to tumors in immunocompetent mice and had a higher rate of rejection by the latter [6, 7]. These studies provided evidence for cancer immunoediting by suggesting that immune
deficiencies allowed persistence of tumor antigens that would have been eliminated in an immunocompetent host. Since then several other immune factors have been shown to be involved in modulating growth of transplanted, chemically induced or spontaneous tumors [2]. These include knockouts of cytolytic machinery such as perforin, cytokines in addition to IFN-γ and mice lacking cellular compartments (for example mice treated with anti-CD25 antibody show decreased incidence of chemically induced sarcoma due to an absence of regulatory T cells). These data provide an abundance of evidence that neoplasms are detected and eliminated by the immune system in steady state. However, it is important to note, that a number of KO mice where tumor immunity is affected are combined deficiencies (say of two cytokines). This reflects the redundancy in the immune response to tumors and may explain early negative data. It also suggests that anti-tumor immunity is an evolutionarily important and conserved process.

**Mechanisms of protective immunity**

Several pathways have been implicated in generating protective immunity against tumors. While the data depends on the murine model used, some mechanisms can be generalized. With respect to innate mechanisms, cancer cells are sensitive to cytokines such as IFN-γ and tumor necrosis factor alpha (TNF-α). These cytokines induce growth arrest and apoptosis in tumor cells. Innate cell types such as natural killer (NK) cells are known to respond to stress ligands expressed by tumors. However, these pathways, while good targets for therapy, would have to rely on bystander stimulation of an adaptive response for immune memory.
Tumors can induce both a CTL and an antibody response. Priming of the anti-tumor immune response to tumor antigens occurs in the tumor draining lymph node. Tumor antigen can drain directly into lymph nodes or be carried by dendritic cells (DC). Tumor antigen is believed to be derived from apoptotic and necrotic tumor debris, or debris from components of the tumor microenvironment such as the endothelium. Exosomes may be another way by which tumor antigen is taken to the draining lymph node. DC migrating from the tumor can be immunosuppressive (see “Mechanisms of immune evasion by tumors”) or express costimulatory ligands and cytokines. Stimulation of DC can occur due to recognition of endogenous adjuvants released from necrotic cancer cells such as HMGB1.

Patient immune responses are typically detected using serum for antibody and peripheral blood mononuclear cells (PBMC) for T-cell responses but some studies have looked at the immune response at the tumor site. Some of the most convincing data to support immune mediated tumor destruction comes from the now numerous studies showing a correlation of increased T-cell function (in most cases CD8) at the tumor site with improved prognosis in patients treated with either conventional cancer therapy [8] or immunotherapy [9]. Several groups have isolated anti-tumor CD8, CD4 and antibodies from patients. As expected, CD4 and CD8 target a diversity of antigens, including novel epitopes generated due to mutations in the cancer cells. Surprisingly, anti-tumor antibodies seen in patients target both surface and intracellular antigens. One scenario that can be envisioned is that tumor blebs are internalized by B-cells and intracellular antigens presented to CD4. This would allow crosstalk between the B-cell and CD4
leading to class switching. The functional importance of antibodies detected to intracellular antigens is not clear. Review articles, in fact, often suggest that anti-tumor antibody responses are not clinically effective. However, these reviews have not taken the latest data into account. Studies have now shown that patient outcome correlates with antibody responses to stress ligands and angiogenic factors expressed by the tumor [10, 11].

Mechanisms of immune evasion by tumors

For simplicity, the pathways employed by cancer to evade the immune system can be categorized as follows:

1. Loss of antigenicity: Cancer cells often downregulate antigen presentation or expression of specific antigens. This phenomenon is called immune editing.

   Further, “creative” strategies such as decoy soluble antigens or use of proteases to cleave extracellular regions of surface proteins are often seen in patients.

2. Expression of inhibitory ligands: Cancer cells have high surface expression of inhibitory ligands such as PD-L1 to suppress lymphocytes in contact with cancer cells.

3. Soluble factors that suppress or kill anti-tumor leukocytes: An example of this strategy is the overexpression of the enzymes indoleamine 1,3-dioxygenases 1 and 2 which generate metabolites toxic to the lymphocytes. The systemic and tumor site cytokine milieu (for example, the presence of IL-10) is also often suppressive to myeloid cells making them poor at activating T cells.

4. Induction of suppressive cell types to restrict anti-tumor lymphocytes: Patient and
murine models both show systemic and tumor site abundance of suppressive cell types such as regulatory T-cells and myeloid derived suppressive subsets. In fact tumor infiltration of regulatory T-cells is a marker of poor prognosis in several cancers.

5. Depletion of metabolic or structural elements essential for lymphocyte function: Solid tumors are often hypoxic and lymphocyte survival and function is markedly depressed under such conditions. Nutrient depletion such as loss of arginine caused by arginase also leads to reduced lymphocyte function.

It is important to emphasize that several of these mechanisms may be active in a single tumor and/or patient. The above examples are not exhaustive and new mechanisms of immune evasion are discovered frequently. Blockade of such tumor driven “checkpoints”, is a vibrant area of preclinical and clinical development of immunotherapy.

**Cancer promoting effects of the immune system**

Cancers hijack the proliferative and angiogenic effects of the anti-tumor immune response. Cytokines, extracellular matrix modifying enzymes, as well as angiogenic factors produced by recruited immune cells promote tumor growth. These are important considerations when designing immunotherapy especially systemic cytokine delivery. However, tumor promoting effects have not been seen in cancer vaccine trials.

Conversely, chronic inflammation can lead to increased incidence of malignancy [12].
Examples include H. pylori infection, obesity and some autoimmune disorders. Pathogen or danger associated molecular pattern (PAMP or DAMP) receptors have been implicated in the initiation of chronic inflammation. PAMP and DAMP induced cytokines such as TNF-α connect chronic inflammation and neoplasia. Further downstream, NF-kB and STAT-3 are examples of transcription factors induced by chronic inflammation in immune and non-immune cells. How does chronic inflammation promote malignancy? One explanation based on the above observations is the induction of a hyper-proliferative state in the affected tissue. However, inflammation can directly increase the rates of mutagenesis by downregulation of mismatch repair genes and the production of reactive oxygen and nitrogen species which are known to cause mutations [12]. Many inflammatory cytokines induce miR-155, which targets the mismatch repair pathway [13]. Activation linked cytidine deaminase (AID), an enzyme causing DNA breaks, previously thought to be expressed only in B-cells, has now shown to be induced in intestinal epithelium in response to inflammation.

**Current status of tumor immunotherapy**

The existence of tumor associated antigens, the ability of the immune system to mount an anti-tumor immune response and paradoxically even our knowledge of the mechanisms of immune evasion, all suggest that tumor immunotherapy is a feasible strategy. The promise of immunotherapy is tumor-restricted cytotoxicity and induction of immune memory; such that a cure for cancer can be envisioned instead of just prolonging patient life with the debilitating effects of conventional therapies.
In 2011, Ipilimumab became the second cancer immunotherapy to be approved by the
FDA [14]. The first was a cell based therapy with very modest effects offset by extremely
high cost. Ipi, on the other hand, is remarkable as it is a monoclonal antibody with wide
applications and provides proof of principle for “checkpoint blockade” in patients. It is an
antibody that blocks the function of a coinhibitory receptor, CTLA-4, which is highly
expressed on tumor infiltrating lymphocytes. It is the first therapy to show an
improvement in survival in metastatic melanoma patients. Several clinical trials are in
progress testing blockade of other checkpoints such as the interaction between T-cell
coinhibitory molecule PD-1 and its ligands PD-L1 and 2. Other monoclonal antibodies
targeting surface expressed proteins include Herceptin (anti-Her2) and Campath (anti-
CD52). However, these were not developed as immunotherapies but merely as reagents
to block pro-survival signals from the respective tumor expressed targets.

Collectively, in the last two decades, the field of tumor immunology has generated
several “druggable” targets with the advantage of being applicable across many cancers.
While checkpoint blockade has received the most press, other promising
immunotherapies include

1. Chimeric antigen receptor (CAR) expressing T cells: Anti-tumor T-cells from
   patients can be expanded ex-vivo and re-infused. In recent years, they have been
   engineered by recombinant DNA technologies to express chimeric receptors on
   their surface allowing recognition of tumor antigens and activation of the
cytolytic machinery independent of the specificity of the T-cell receptor. CAR
   allow targeting of the bulk T-cells against the tumor [15].
2. Whole cell vaccination: Whole tumor cells have been used to stimulate anti-tumor immune responses in patients in combination with cytokines and adjuvants [16]. Whole tumor cells allow a non-biased and robust presentation of tumor antigen in the presence of pro-inflammatory signals. Clinically one of the adjuvants tested has been BCG and others are being tested in animal models. None of these immunotherapies are approved in the US. Amongst the whole cell therapies currently in trial, GVAX appears to be the most promising [15].

3. Novel biomaterials to deliver immunotherapies: One approach that has received a lot of attention is the use of biomaterials to enhance immunotherapy. Nanoparticles can be used to conjugate cargo to immune cells, deliver adjuvants or antigens to lymph nodes or provide the right-milieu for recruited myeloid cells. The only example of such an approach in clinical trial is a non-cellular form of GVAX (http://directorsblog.nih.gov/2013/09/17/personalized-cancer-vaccine-enters-human-trials). Patient tumor lysate is loaded in a dime sized bioabsorbable porous disc along with GM-CSF and adjuvants to provide costimulation in a 3D structure to responding antigen presenting cells.

Advantages of tumor immunotherapy over conventional therapies

Mice responding successfully to immunotherapy show long-term protection against a re-challenge with related tumors. Immunotherapy, hence, promises a cure rather than a reprieve. Most immunotherapy leads to responses against multiple antigens. This lowers the probability of escape variants as seen in conventional monotherapies. Immunotherapy is also the only therapeutic approach available with the potential to be absolutely specific
to the tumor. While this ideal has not been realized, side effects of immunotherapy are relatively limited compared to chemotherapy or radiation.

It is important to consider here the one major disadvantage of some immunotherapeutic approaches: that of cost. All cell-based immunotherapies require extensive ex-vivo cell manipulation and are prohibitively expensive. However, other therapies such as monoclonal antibodies are comparable in cost to conventional cancer therapy. Critics of tumor immunology also point to the large number of failed clinical trials of cancer immunotherapies. But, cancer clinical trials, conducted in very sick patients and often after several previous treatment modalities, have an extremely high rate of failure irrespective of whether the trial is evaluating chemotherapy or immunotherapy. Moreover, while immunotherapies are generally tested as single agents, the diverse mechanisms of immune evasion suggest that success of cancer immunotherapy likely depends on combinatorial approaches.

Combinatorial strategies in preclinical and clinical development include “active immunotherapy” combined with checkpoint blockade (e.g. GVAX+Ipilimumab [17]) or a combination of immunotherapy and chemotherapy (vemurafenib, a small molecule inhibitor of a B-raf mutation seen in some melanoma patients and Ipilimumab [18]). “Active” immunotherapy refers to immunization against tumors. In mice treated with GVAX, CTLA-4 appears to restrain the immune response [19]. This appears to be a generalizable phenomenon and suggests that active immunotherapy would benefit from additional strategies to combat immune evasion.
Recent data for radiotherapy, several types of chemotherapies (namely, anthracyclines, gemcitabine and lenalidomide) and the targeted therapy imatinib, all suggest that conventional anti-tumor therapies work, in part, by activating the immune system. This provides a convincing rationale for combination of chemotherapy/radiotherapy with immunotherapy. In addition, clinicians hope to be able to reduce chemotherapy doses and toxicity, yet take advantage of the rapid chemotherapy induced tumor destruction by combining it with immunotherapy.

**GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR**

GM-CSF is a 15 kD secreted protein produced by immune cells such as T-cells, mast cells and basophils; and non-immune cells such as endothelial cells (reviewed in [20, 21]) and keratinocytes [22-24]. The receptor for GM-CSF is expressed on all myeloid lineages, hematopoietic progenitor cells and endothelial cells. GM-CSF first binds a specific α-subunit of the receptor, which in turn engages a β-subunit that is common to GM-CSF, IL-3 and IL-5 receptors [25].

GM-CSF was identified as a factor that promoted the in-vitro differentiation of monocytes and granulocytes from mouse bone marrow [26]. It is widely used in the laboratory to induce murine bone marrow derived dendritic cells as a single agent and in various cytokine cocktails to induce human dendritic cell (DC) subtypes. In the absence of additional signals, DC derived *in-vitro* with GM-CSF are immature and induce T-cell suppression and/or induction of regulatory T-cells (Treg). Additional signals such as cytokines or pathogen associated molecular patterns (PAMP) lead to activated DC that
can stimulate T cell responses [27]. This mirrors the in-vivo roles of GM-CSF (as elucidated by the GM-CSF deficient mice; discussed below) where defects in dendritic cells can cause inflammation due to loss of immunomodulatory functions such as induction of Treg. Yet, loss of dendritic cells also leads to a deficiency in induction of protective immunity in models of infectious disease. The phenotypes of GM-CSF KO and the functions of GM-CSF in the various immune subsets will be discussed in-depth the following sections.

**Effect of GM-CSF deficiency on lymphoid dendritic cell subsets in naïve mice**

Lymphoid tissue dendritic cells can broadly be divided into two functional groups by the expression of CD8α. CD8α+ dendritic cells are considered to be tolerogenic unless activated. On activation, they can efficiently cross-present antigens to CD8 T cells. CD8α- DC appear to be more specialized for activating CD4 T cells.

GM-CSF KO mice show negligible to modest reduction in lymphoid DC [28]. This was surprising given the potent DC differentiation induced by GM-CSF in-vitro. Kingston et al. [29] reasoned that this may be due to redundant functions of GM-CSF and another DC promoting cytokine Flt3L. FLt3L KO mice have severe DC defects, which are modestly enhanced in naive GM-CSF/Flt3L DKO mice.

**Effect of GM-CSF deficiency on inflammatory monocyte derived DC**

An expansion of activated monocytes and monocyte derived dendritic cells usually accompanies an immune response to pathogens. There are conflicting studies on the role
of GM-CSF in inducing inflammatory monocyte derived dendritic cells [27, 30].

Inflammatory monocyte derived DC are specialized for producing TNF-α and iNOS and are called TIP-DC. In this capacity they are important for early anti-microbial responses. They are also known to stimulate potent helper T-cell responses in various models of infectious disease.

GM-CSF deficiency reduces protective immunity against many but not all pathogens. It’s not clear if it is preexisting DC or inflammation induced monocyte derived DC that contribute to loss of protective immunity in GM-CSF KO mice.

**Effect of GM-CSF deficiency on non-lymphoid dendritic cell subsets**

Non-lymphoid DC can broadly be divided into CD103+ and CD103- cells. CD103+ and CD103- cells are evolutionary counterparts of CD8+ and CD8- lymphoid DC and display similar functional compartmentalization. Greter et al. [30] found marked reductions in CD103+ DC in the lung and intestines of GM-CSF KO mice. Loss of GM-CSF in the intestinal mucosa leads to disruption of homeostasis and inflammation due to the lack of DC and secondary effects on Treg. Similar effects have been seen for skin dendritic cells [29-32].

These data indicate a non-redundant function of GM-CSF function at mucosal barriers. GM-CSF KO mice are more susceptible to tuberculosis and influenza. Effect of GM-CSF dependent dendritic cells on host immunity to lung pathogens is challenging to study due to independent defects in alveolar macrophages [33]. Nonetheless, it is clear from other
genetic models where CD103+ DC are reduced that they are important in mounting an immune response to influenza [34] and tuberculosis [35]. Reduced number of intestinal DC also leads to increased susceptibility of GM-CSF KO mice to enteric pathogens [36].

Interestingly, the two main phenotypes to emerge from the GM-CSF KO are functional defects in phagocytosis [6, 31] and could not have been predicted a priori based on GM-CSF functions in-vitro.

**GM-CSF and phagocytosis of apoptotic cells**

Enzler et al. found that GM-CSF KO macrophages are deficient in the uptake of apoptotic cells [6]. Jinushi et al. further showed that this defect was seen in several myeloid populations including lymphoid dendritic cells and was due to defects in expression of proteins such as MFG-E8 that bridge apoptotic cells and phagocytes [37]. The defect in the clearance of apoptotic cells leads to chronic inflammation and autoimmunity. An autoimmune lupus-like disorder is seen in aged GM-CSF KO mice.

**GM-CSF and phagocytosis and catabolism of surfactant**

The most striking phenotype of adult GM-CSF KO mice is lung inflammation due to reduced phagocytosis of surfactant by alveolar macrophages [31]. Surfactant is an emulsion of phospholipid and protein, lining the alveolar spaces. In KO mice, surfactant builds up in the lung and in the macrophages, causing cell death and inflammation. In fact, this phenotype is a faithful model of a disease seen in patients with reduced GM-CSF function, called pulmonary alveolar proteinosis. GM-CSF induces the expression of
transcription factors PU.1 and PPAR-γ in alveolar macrophages. Together they control many proteins involved in binding, uptake, catabolism of surfactant proteins and phospholipids [16, 38].

**GM-CSF and Treg**

The autoimmunity in GM-CSF KO mice is correlated with reduced regulatory T-cell numbers in the periphery. Wild type phagocytes repress inflammation on phagocytosis of apoptotic cells in part through the production of TGF-β and induction of Treg. GM-CSF KO phagocytes are deficient in inducing TGF-β on apoptotic cell phagocytosis and this defect could be contributing to the reduced Treg numbers. GM-CSF induced expression of inhibitory ligands such as PD-L1 [35] and Ox40L [39] on dendritic cells can also lead to expansion of regulatory T-cells. GM-CSF can also induce retinoic acid production by dendritic cells [40] and it has been shown that retinoic acid can promote regulatory T-cell induction. A loss of retinoic acid and Treg has been noted in the intestinal mucosa of GM-CSF KO mice and has been linked to an increased susceptibility to chemically induced colitis. It is important to note that the effect of retinoic acid, namely effector T-cell differentiation or regulatory T-cell differentiation is a matter of debate and may be modulated by additional signals such as TLR ligation received by the antigen presenting cell (APC) [41].

**GM-CSF in cancer**

Several solid tumors express GM-CSF. It has been shown that GM-CSF is a potent inducer of suppressive phenotypes in tumor associated and peripheral monocytes and
granulocytes. These suppressive abilities include induction of Treg and suppression of effector T cell function by depletion of extracellular arginine. GM-CSF, not surprisingly, is a growth factor for myeloid leukemias (reviewed in [25]).

**GVAX**

Clearly, GM-CSF can have context dependent effects on dendritic cells leading to immunosuppression or stimulation. The findings that lead to the use of GM-CSF in the context of immunotherapy predated the studies revealing its immunosuppressive role. In the B16 melanoma model (reviewed later), whole cell vaccination, even prophylactic, doesn’t induce protective anti-tumor immunity. Dranoff et al. asked which cytokine could induce anti-tumor immunity in prophylactic whole cell vaccination. Of the 7 cytokines tested, GM-CSF was one of the few to generate protective immunity and the only one to show dramatic reduction in tumor incidence and improvement in survival [42].

**GVAX clinical trials**

GVAX as a single agent induces robust immune infiltrates at the vaccine site and distant metastases. Patients show tumor specific T-cells and antibody responses. Histological analysis of metastases show a T-cell response localized with dying tumor cells. While the early single agent trials were not designed to evaluate survival, some of the patients show remarkable regressions and improvement in survival. Trials were conducted with using autologous cells [43-46] [47, 48] or allogeneic cell lines to obviate the need for patient cell collection and manipulation [48, 49]. Some investigators tried a mix of patient cells and allogeneic cell lines to provide personalized tumor antigens without having to
transduce primary cells.

A phase III trial of allogeneic GVAX for prostate cancer did not show any survival benefit. Expert opinion is divided as this trial tested GVAX in combination with chemotherapy which can reduce immune function [49]. However, other trials of GVAX in combination with chemotherapy have shown promise [50, 51].

The understanding that GM-CSF may play immunosuppressive roles and that patient immune dysfunction could be reversed by strategies such as checkpoint blockade lead to modified trial design where GVAX was tested in combination with other immunotherapies [52] including hematopoietic stem cell transplantation [53] and CTLA4 blockade [9].

GVAX has been instrumental in delineating basic concepts in cancer immunotherapy of patients. For instance, trials of GVAX in combination with Ipilimumab, the CTLA-4 blocking antibody, revealed that with prior immunotherapy, the dose of CTLA-4 blockade needed and consequent toxicities could be reduced. Further, this trial substantially added to the understanding that skewing the balance in favor of cytolytic cells at the tumor site is important in leading to tumor regression. The study corroborated early data that an improved ratio of CD8 T-cell to regulatory T-cells at the tumor site correlates with improved anti-tumor immunity [9].
**Murine model of GVAX**

The murine model most widely used for GVAX and studied in this manuscript is the B16 melanoma model. Vaccination with irradiated wild-type B16 induces no protection against a subsequent challenge with live B16, suggesting profound immunosuppression. If GM-CSF expressing B16 are used as a vaccine, then 100% of the mice can reject a subsequent challenge with live wild type B16. As mentioned earlier, of the 7 cytokines tested, only GM-CSF had such a marked anti-tumor effect [42].

GVAX is administered as a subcutaneous injection. While trafficking of DC subsequent to GVAX injection has not been studied, other models of subcutaneous injection suggest that epidermal and dermal DC traffic to draining lymph node to induce an immune response. In this regard it is important to note that there are contradictory reports of CD103+ dermal DC survival being affected in GM-CSF KO mice. Modest effects on Langerhans cells which are the epidermal DC have been noted in GM-CSF deficient mice. ([29-32], Dranoff et al.unpublished data)

In the murine model, CD8 depletion abrogates GVAX efficacy. Adoptive transfer of CD8 is enough to confer protection. However, this does not imply that the endogenous CD8 response is sufficient to induce protection to GVAX. Previously NK and NKT [54] cells have also been shown to be required for full GVAX efficacy. Our group has also shown that GVAX induces an anti-tumor antibody response in mice (Joo Sang Park unpublished data, [55]). The role of T-helper cells appears to be context dependent. CD4 T-cells are required in a model of prophylactic vaccination using irradiated B16-GM-CSF cells [56]
but not when live B16-GM-CSF cells are used [57]. In CD1d KO mice, where GVAX does not protect against tumor challenge, the primary defect appears to be a lack of Th2 cytokines [54]. These data coupled with the induction of an antibody response suggest that CD4 are in fact important in GVAX.

Given that GM-CSF can induce FoxP3+ regulatory T-cells ex vivo and KO mice have a defect in this compartment, investigators have tested the how Treg depletion impacts GVAX. Depletion of Treg using CD4 or CD25 antibody causes significant improvement in the efficacy of GVAX [57]. Thus, GVAX efficacy can be improved by limiting its immunomodulatory functions. In fact, GVAX shows significant synergy with T-cell checkpoint blockade in a number of murine studies and clinical trials [9]. Ipilimumab, was extensively validated in combination with GVAX during preclinical studies [58]. Similarly, PD-1 blockade synergizes with GVAX to improve survival in murine models.

**GM-CSF AND PPAR-γ**

PPAR-γ is a nuclear receptor whose expression is maintained in alveolar macrophages by the presence of GM-CSF. PPAR-γ was found to be reduced in alveolar macrophages from pulmonary alveolar proteinosis patients [16]. These patients are known to have reduced GM-CSF function. Detailed studies in mice and humans have shown that GM-CSF induced PPAR-γ is essential in surfactant phagocytosis and catabolism by alveolar macrophages. Pulmonary proteinosis seen in GM-CSF KO mice can be rescued by reintroducing PPAR-γ expression using adenoviral vectors [59].
**PPAR-γ introduction**

PPAR-γ is a nuclear receptor which responds to lipid ligands. It was first identified as the master regulator of adipogenesis [60]. Amongst other roles, PPAR-γ induces pathways involved in storage of triglycerides in adipose tissue. Counterintuitively, PPAR-γ is best known for its ligand, Avandia or rosiglitazone (Rosi), which is a diabetes drug. In the past, PPAR-γ’s effects on glucose metabolism in adipose tissue, muscle and liver were proposed as mechanisms to explain why activation of a pathway that promotes storage of triglycerides could lead to lower insulin resistance. As discussed later, recent preclinical data suggest that PPAR-γ function in immune subsets contributes to the anti-diabetic effects of Rosi.

PPAR-γ and related family members can either directly activate or repress transcription or inhibit transcriptional activation by another transcription factor, a process termed transrepression. For direct activation or repression, PPAR-γ is an obligate heterodimer with members of the retinoid X-receptor family (RXR). Both PPAR-γ and RXR need to bind their ligands to activate the locus instead of repressing it. It is not clear if PPAR-γ mediated transrepression requires heterodimerization with RXR.

In the immune system, PPAR-γ expression has been shown in T cells, B cells and myeloid cells. Immune mediators such as the prostaglandins have been proposed as PPAR-γ ligands though endogenous ligands have not been conclusively defined. Most studies use synthetic ligands from the thiazolidinedione family (same family as Rosi).

CD4-Cre; PPAR-γ KO mice show enhanced Th17 induction in an autoimmune
encephalitis model due to greater expression of ROR-\(\gamma\)T, the transcription factor controlling Th17 differentiation [61]. These data were preceded by many studies showing that pharmacological activation of PPAR-\(\gamma\) leads to increased apoptosis in murine and human T-cells. Interestingly, siRNA mediated knockdown of PPAR-\(\gamma\) in human CD4 T-cells also increases Th17 differentiation but only in CD4s from female donors [62]. Given that ROR-\(\gamma\)T and FoxP3 are mutually antagonistic, it is interesting to note that one study has shown a role for Treg intrinsic PPAR-\(\gamma\) expression in Treg maintenance in adipose tissue [15].

In B-cells, PPAR-\(\gamma\) is required to initiate a differentiation program upon activation. PPAR-\(\gamma\) KO B cells do not upregulate activation markers and do not participate in germinal center reactions leading to reduced antibody responses to the model antigen, ovalbumin [45].

In macrophages, PPAR-\(\gamma\) can act by directly inducing transcription of target genes (CD36, arginase I) or antagonizing NF-kb transcriptional activity to suppress production of inflammatory cytokines such as TNF-\(\alpha\), IL-6 and IFN-\(\gamma\). Repression of NF-kb transcriptional activity correlates with observed defects in pathogen clearance and increased induction of autoimmunity in LysM-Cre; PPAR-\(\gamma\) fl mice. LysM-Cre; PPAR-\(\gamma\) fl mice phenocopy GM-CSF KO mice; not only in proteinosis as mentioned above, but also in phagocytosis of apoptotic cells leading to autoimmune disease even in naïve mice. Similar phenotypes are seen in LysM-Cre; RXR\(\alpha\) fl mice suggesting that it is the PPAR-\(\gamma\)/RXR\(\alpha\) heterodimer that mediates GM-CSF mediated homeostatic apoptotic cell clearance [63].
PPAR-γ has also been suggested to be required for alternative activation of macrophages. PPAR-γ expression is highly induced by IL-4 and is required for M2 pathways such as arginase I induction. Genes positively regulated by PPAR-γ include those characteristic of alternatively activated macrophages such as Fizz1 and mannose receptor. Given GM-CSF function, PPAR-γ could be playing a role in DC differentiation. Yet so far no differentiation defects have been reported using genetic depletion. With the exception of one study, most investigators find that treating DC with PPAR-γ ligand suppress their ability to activate T cell responses.

PPAR-γ is a member of a family of nuclear receptors. Related receptors such as PPAR-δ and downstream effectors such as LXR-α and β have been studied in immune cells and often recapitulate the functions associated with PPAR-γ [64].

**Crosstalk between PPAR-γ’s metabolic role and immune functions**

Studies show that differentiated but resting myeloid cells rely on beta-oxidation (use of triglycerides as energy sources) compared to activated myeloid cells which switch to glycolysis. Given the recent interest in metabolic states of immune cells, the role of metabolic changes induced by PPAR-γ in immune cells should be of interest. Yet, so far, PPAR-γ’s metabolic activities have not been linked to myeloid function. The reverse is however true, PPAR-γ’s immune functions appear to impact metabolic homeostasis. LysM-Cre; PPAR-γ fl mice display chronic inflammation and insulin resistance upon a high fat diet [65]. These data suggest that the effect of PPAR-γ activating diabetes drugs
on limiting chronic metabolic diseases could be mediated through maintaining myeloid homeostasis. Interestingly, in another example of crosstalk, a recent paper shows that the apoptotic cell binding protein MFG-E8 (deficient in both LysM-Cre; PPAR-γ fl mice and GM-CSF KO mice) can also mediate phagocytosis of fatty acids [66].

**PPAR-γ and cancer**

Our study is the first to our knowledge to use a conditional KO to study the role of PPAR-γ in the immune response to tumors. Given PPAR-γ function in inducing alternative activation of macrophages, it would be interesting to see if any role of PPAR-γ emerges in tumor associated macrophages in future studies.

Many groups have studied tumor cell intrinsic functions of PPAR-γ. In a majority of cases, though not all, PPAR-γ activation appears to be detrimental to tumor survival. In fact, thyroid carcinomas are known to have chromosomal translocations leading to expression of mutated PPAR-γ that exerts a dominant negative effect. ~30% of follicular thyroid carcinomas have a PAX8-PPAR-γ translocation. PPAR-γ ligands are being investigated as chemotherapeutic agents. As a monotherapy, PPAR-γ activation has not lived up to its preclinical promise in patients. Yet, there are encouraging preclinical data that show significant anti-tumor effects of PPAR-γ ligation in combination with platinum based chemotherapy or retinoic acid treatment [67]. In preclinical studies of PPAR-γ as a chemotherapeutic agent, the effects on the immune system were not investigated even when immunocompetent mice were used.
HYPOTHESIS

Table 1 compares the phenotypes of the GM-CSF KO and the LysM-Cre; PPAR-\(\gamma\) fl mice. The overlap between the two is striking. The strong genetic link in the lung and the similarities between the KOs lead us to explore the role of myeloid PPAR-\(\gamma\) in GVAX. Given PPAR-\(\gamma\) mediated transrepression of NF-\(\kappa\)b and its role in maintaining an immunological homeostasis, we predicted that GVAX efficacy would increase in LysM-Cre; PPAR-\(\gamma\) fl mice.

Table 1: Comparison of phenotypes observed in GM-CSF KO and LysM-Cre; PPAR-\(\gamma\) fl mice

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Observed in GM-CSF KO</th>
<th>Observed in LysM-Cre; PPAR-(\gamma) fl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surfactant accumulation</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Proteinosis</td>
<td>Severe</td>
<td>Modest</td>
</tr>
<tr>
<td>Defective phagocytosis of apoptotic cells</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Defective expression of MFG-E8</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Increased Th1</td>
<td>Yes</td>
<td>Not investigated in B6; Yes in Balb/c</td>
</tr>
<tr>
<td>Decreased Treg</td>
<td>Yes</td>
<td>Not investigated</td>
</tr>
<tr>
<td>Autoimmune disease</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
REFERENCES


Chapter 2

The role of GM-CSF in maintaining PPAR-γ expression in myeloid and non-myeloid populations

INTRODUCTION

The first set of studies that we conducted before testing the role of PPAR-γ in GVAX was to identify which tissues in steady state require GM-CSF for PPAR-γ expression. These analyses were focused on myeloid cells though relevant non-myeloid populations were also tested.

GM-CSF has previously been shown to be necessary for the expression of PPAR-γ in alveolar macrophages. Alveolar macrophages perform a unique function, in catabolizing surfactant which is dense in lipids and proteins. No other cell type is known to process the estimated levels of lipids under steady state except adipocytes which store the triglycerides. In pathological conditions, macrophages in atherosclerotic plaques may also phagocytose large amount of lipids. PPAR-γ is implicated in lipid uptake, processing and storage in all three cell types mentioned above. Thus, an evolutionary argument can be constructed for the regulation of PPAR-γ in alveolar macrophages.

We wondered if PPAR-γ is expressed in cell types other than alveolar macrophages in steady state and if this expression requires GM-CSF. As discussed in the introductory chapter, GM-CSF and PPAR-γ have both been implicated in phagocytosis of apoptotic cells. How the lipid content of apoptotic cells, especially the significant membrane fraction, is processed by phagocytes is unknown. In the studies in GM-CSF KO and LysM-Cre; PPAR-γ fl mice, a defect in phagocytosis of apoptotic cells was shown in several myeloid subsets from various anatomical
locations suggesting an important role for PPAR-γ in anatomically and functionally distinct myeloid populations.

PPAR-γ has two full-length isoforms (Fig 1, modified from reference [1]). The functional difference between the isoforms is not clear though the adipogenic potential of PPAR-γ2, the longer isoform with 30 additional amino acids at the N-terminus, is greater. For these studies, we have compared C57Bl/6J mice to mice doubly deficient in GM-CSF and IL-3. GM-CSF and IL-3 share a receptor subunit and it is suspected that studies of GM-CSF deficiency may be confounded by increased receptor availability to IL-3. There is no known link between IL-3 and PPAR-γ.

![Figure 1: Isoforms and domains of full length PPAR-γ [1].](image)

**Study Objectives**

1. To develop an assay to detect PPAR-γ

2. To define tissue specific dependence of PPAR-γ expression on GM-CSF function and to confirm previously published connection in alveolar macrophages.
METHODS

Virus generation

cDNA encoding for each isoform of PPAR-γ was inserted into the retroviral vector pMFG using standard recombinant DNA technology. pMFG-PPAR-γ plasmid was transfected into a packaging cell line, 293GPG, which expresses the protein components necessary for viral assembly using Lipofectamine. Supernatants containing the secreted virus were collected starting on day 2 for several days. Virus particles were precipitated by high-speed ultracentrifugation, resuspended in OptiMem and stored in -80°C till needed.

B16 culture and infection

B16 were cultured in DMEM containing 10% FCS and antibiotics. For infection, 1X10^5-2X10^5 B16 were plated and incubated with polybrene and concentrated virus. After 24 hours, cultures were washed and allowed to become confluent.

Lysate preparation and Western Blot for detection of PPAR-γ protein

Depletion of CD11b cells was performed using magnetic beads (Miltenyi Biotec). Cells were lysed in the following: RIPA buffer containing 10% protease inhibitors (Sigma-Aldrich; Cat. No. P8340) and ImM Na3OV4 and PMSF. Immediately after lysis, samples were sonicated briefly and then spun down at 15000g for 15 min at 4°C. Supernatants were collected and heated to 70°C with loading buffer containing lithium dodecyl sulfate and 100mM DTT.

For Western Blotting, a rabbit anti-PPAR-γ antibody (Cell Signaling, 81B8) was used followed by an alkaline phosphatase conjugated secondary. A chemiluminescent substrate was used to develop the blot (Vector Labs, SK-6605). Densitometry was conducted using ImageJ software.
RESULTS

Detection of PPAR-γ by Western Blot

We required a robust assay to detect PPAR-γ to confirm its relationship with GM-CSF and myeloid specific loss in GM-CSF-/− mice. We generated B16 derived cell lines overexpressing each isoform and screened commercially available antibodies to select one with robust and specific detection of PPAR-γ (Fig. 2). We screened alveolar and peritoneal macrophages; and CD11b+ splenic cells. CD11b is expressed on monocytes and neutrophils. Thus this population includes splenic monocytes, macrophages, monocyte derived dendritic cells and neutrophils. We also tested perigonadal fat pads (as an endogenous positive control), total bone marrow and the bulk spleen left after CD11b fractionation. We were unable to detect PPAR-γ in splenic myeloid cells or freshly recovered peritoneal macrophages. Most importantly, endogenous PPAR-γ was detectable in alveolar macrophages using this antibody (Fig. 2). As reviewed in the introduction, GM-CSF mediates important aspects of alveolar macrophage biology and thus they represent an important cell type to study GM-CSF induced genes.
**Figure 2: Expression of PPAR-γ in B16 cells and various tissues.** Lysates were made from the indicated tissue and analyzed for PPAR-γ expression. β-actin expression for normalization.

**PPAR-γ is a target of GM-CSF in alveolar macrophages**

PPAR-γ expression in alveolar macrophages was previously shown to be GM-CSF dependent. We were able to confirm these findings. Alveolar macrophages from GM-CSF KO mice were completely deficient in PPAR-γ (Fig. 3). Interestingly, we found that freshly isolated peritoneal macrophages did not express detectable amount of PPAR-γ protein. Adherence to a tissue culture dish upregulated expression which was not GM-CSF dependent (Fig. 4). We also tested if thioglycollate elicitation would lead to PPAR-γ expression and its dependence on GM-CSF. Significant but GM-CSF independent PPAR-γ can be detected in peritoneal macrophages upon thioglycollate elicitation (Fig. 5). Further, PPAR-γ expression in perigonadal fat pad and CD11b depleted spleen was also GM-CSF independent (Fig. 6 and 7). Two experiments are shown for perigonadal fat as expression was quite variable from mouse to mouse. These findings suggested that PPAR-γ is a GM-CSF target in certain macrophages and that differentiation or anatomical
location conferred differential dependence on GM-CSF for PPAR-γ expression. In fat and lymphocytes (CD11b depleted spleen) which do not express GM-CSF receptor, PPAR-γ expression is expected to be independent of GM-CSF.

Figure 3: Detection of PPAR-γ protein confirmed a requirement for GM-CSF to maintain PPAR-γ expression in alveolar macrophages. Alveolar macrophages from 2-wk old mice were collected by bronchoalveolar lavage (BAL) to reduce the confounding effects of proteinosis seen in older animals. The entire contents of the BAL from each mouse were lysed and loaded in a single lane. 3 WT and 3 GM-CSF-/- animals are shown above and B16 cells transduced with each of the two PPAR-γ isoforms were used as positive controls.
Figure 4: PPAR-γ expression in resting peritoneal macrophages 5 hours after plating.

Peritoneal cells were collected by a lavage and then plated for 5 hours. Non-adherent cells were washed off and the adherent cells were lysed in situ. Each lane represents one mouse.
Figure 5: PPAR-γ expression in thioglycollate elicited peritoneal macrophages. Peritoneal cells were collected by a lavage and CD19 depleted. Remaining cells were lysed and lysates from individual mice were loaded in each lane.
Figure 6: PPAR-γ expression in perigonadal adipose tissue. Adipose tissue was mechanically crushed in lysis buffer to obtain the lysate. Each lane represents an individual mouse.

Figure 7: PPAR-γ expression in CD11b depleted splenocytes. Each lane represents an individual mouse.
We also tested if PPAR-\(\gamma\) expression could be detected by flow cytometry. We were able to detect ectopic expression in B16 (Fig. 8) but were unable to detect endogenous expression in alveolar macrophages.

**Figure 8: Detection of PPAR-\(\gamma\) by flow cytometry.** A. Detection of overexpressed PPAR-\(\gamma\) in B16 cells. B. Detection of endogenous PPAR-\(\gamma\) in alveolar macrophages.

**DISCUSSION**

The above studies are a systematic analysis of PPAR-\(\gamma\) expression in various myeloid as well as selected non-myeloid tissues. Given the defects in apoptotic cell phagocytosis of various myeloid populations deficient in PPAR-\(\gamma\), it is surprising that we were only able to detect PPAR-\(\gamma\)
expression in alveolar macrophages. We find that homeostatic PPAR-γ expression in alveolar macrophages is entirely dependent on GM-CSF presence. GM-CSF has important roles to play in mucosal surfaces, thus it is possible that myeloid populations in the gut mucosa also show GM-CSF dependent PPAR-γ expression. Our data are in accordance with a recently published analysis of PPAR-γ mRNA expression in various myeloid subtypes [2]. Gautier et al also found that peritoneal macrophages only expressed PPAR-γ under inflammatory conditions. To our knowledge, this is the first evidence of adherence leading to PPAR-γ expression in macrophages. Gautier et al also show that monocytes recruited to the site of inflammation express PPAR-γ. Thus, in the following chapter we tested if the immune response to GVAX was affected in LysM-Cre; PPAR-γ fl mice.

REFERENCES


Chapter 3

Effect of genetic loss-of-function in the monocyte lineage on GVAX: studies on candidate mechanisms

INTRODUCTION

Modeling GVAX using the B16 melanoma cell line

GVAX was originally tested in the B16 transplantable tumor model. Efficacy has also been established in several other models. However, unlike other tumor models, immunization with irradiated B16-WT does not impact subsequent growth of live B16. Thus, the role of GM-CSF and its downstream effectors can be studied without the confounding effects of radiation. This reduced immunogenicity is characteristic of the derivative of B16 cells that we are using (other B16 sublines as well). It is denoted B16-F10. The original B16 melanoma was injected i.v. and lung metastases were allowed to grow. After each “passage” the B16 were harvested and the metastatic experiment repeated. The authors found that with each passage, the B16 sub lines had higher metastatic ability as measured by the number of nodules recovered in the lung. In hindsight, we can postulate that passaging B16 in mice may have led to (a) progressive immunoediting, (b) acquisition of immunosuppressive pathways and (c) selected for expression of molecules that enhance homing efficiency/survival in the lung. The B16-F10 was derived from the 10\textsuperscript{th} in vivo passage [1]. Decades of studies have shown that restraining growth of B16 tumors growing in mice is more challenging than most other tumor models. Thus, the B16-F10 (referred to as B16 here for simplicity) is a particularly relevant model for us because:

1. GVAX has been extensively studied in this model.
2. Because of poor immunogenicity, it is a therapeutically challenging to induce regression. It sets a stringent threshold when considering the success of an immunotherapy.

3. Due to its extensive use, some antigens targeted in the response to GVAX are known and tetramers are available to quantify tumor specific CD8 cells.

**Different regimens of vaccination**

We have utilized three different modes of vaccination, all of which are known to induce a B16-specific immune response and are effective to varying degrees in reducing tumor growth. Efficacy can also be modulated by changing the number of cells used for immunization or tumor challenge.

1. **Prophylactic GVAX:** Prophylactic vaccination can be modulated to achieve 100% protection from tumor challenge. In this setting, vaccine induced responses develop in the absence of tumor-induced immunosuppression.

2. **Therapeutic GVAX:** GVAX given simultaneously or after tumor challenge shows minimal effects at improving survival. B16 cells proliferate robustly *in vivo* and thus by the time adaptive immunity to GVAX is generated, the tumors are well established replicating the situation in cancer patients. New immunotherapeutics are often tested in combination with therapeutic GVAX to see if the immune response can be potentiated.

3. **Immunization with live B16-GM:** Live B16-GM-CSF (live-GM; as opposed to irradiated B16-GM) also protects against a subsequent challenge with WT B16 cells despite growth of the primary GM-CSF expressing tumor. These “vaccine tumors” are rich in myeloid cells [2, 3]. We used live-GM injections to assay the myeloid infiltrate at the vaccine site.
Study objectives

1. Generation of a myeloid cell specific KO of PPAR-γ: Two major cre recombinase expressing mice are available for generation of myeloid cell specific KO: CD11c-Cre or LysM-Cre. CD11c is a marker of all dendritic cells and alveolar macrophages and is also expressed at lower levels on NK cells. Lysozyme M is an enzyme expressed in phagolysosomal compartments in neutrophils, monocytes, macrophages and some dendritic cells. We chose LysM-Cre to delete PPAR-γ. GM-CSF acts on monocyte/macrophages, dendritic cells, and granulocytes, so LysM-Cre induced deletion would impact broader range of cell types relevant to the vaccine.

2. Analysis of impact on GVAX induced immunity and reduction in tumor growth in LysM-Cre; PPAR-γ fl mice (hereafter referred to as “PPAR-γ KO”): the following studies describe the effect on vaccine efficacy in the PPAR-γ KO and investigate candidate mechanisms based on published reports that might explain our observations.

METHODS

Generation of LysM-Cre; PPAR-γ fl

Commercially available LysM-Cre (Jackson Laboratory, 4781) and PPAR-γ fl (Jackson Laboratory, 4584) mice were crossed together. F1 X F1 crosses lead to Cre and fl homozygous animals which were viable and fertile.
Restimulation of splenocytes

Spleens were crushed, subjected to red blood cell lysis and passed through a 70um strainer to obtain single cell suspensions. 2X10^6 cells were plated in 2ml of media with 50,000 irradiated B16 cells. Cytokine levels were measured after 4 days.

Tumor processing

B16-GM tumors were harvested and weighed. Tumors were chopped into 1-3 mm pieces and incubated in media containing 200 units Collagenase IV and 10ug/ml DNAse for 45-75 minutes at 37°C. After incubation, the tissue was pipetted repeatedly and strained with a 70um strainer. A gradient for centrifugation was generated using Optiprep (Sigma-Aldrich). 25ml of a solution containing 0.85% NaCl and 10mM Tricine in distilled water was mixed with another 5ml of distilled water and 8.71 ml of Optiprep. This gradient was layered under media containing the tumor single cell suspension and spun at 400g for 25 minutes at RT with slow deceleration. The interface was collected and analyzed for flow cytometry or used for coculture.

Coculture experiments

Naïve and vaccinated spleens were processed to single cell suspensions. CD8 were selected by using anti-CD8 labeled magnetic beads. Following that CD4 were recovered by negative selection, again using magnetic beads. 50,000 APC were incubated with 500,000 CD4 or CD8. For NKT cell coculture, 50,000 APC were incubated with 50,000 24.8 or primary NKT from Vb7 somatic nuclear transfer mice. All CD4 in these mice are NKT cells. There are also CD4-NKT cells. To purify the primary NKT, a negative selection was performed for CD4 using
magnetic beads. For aGC loading, APC were incubated with 500ng/ml aGC for 2-4 hours and then washed repeatedly.

RESULTS

*LysM-Cre; PPAR-γ fl mice show significant loss of PPAR-γ and recapitulate the lung pathology of GM-CSF KO mice*

We crossed LysM-Cre mice to mice with loxP sites flanking the *pparg* locus. As shown in Fig. 9, peritoneal macrophages from PPAR-γ KO mice had a greater than 90% reduction in PPAR-γ protein expression. PPAR-γ KO mice showed some evidence of protein accumulation and inflammation in the BAL, and histologic analysis revealed mild pathologic changes consistent with pulmonary alveolar proteinosis (data not shown). It has been previously shown that proteinosis in PPAR-γ KO mice is not as severe as in GM-CSF deficient mice implying that PPAR-γ is only one of the downstream effectors involved in GM-CSF regulated surfactant homeostasis [4].
Figure 9: Generation of myeloid specific KO of PPAR-γ. Peritoneal lavage was collected and plated for 2-4 hours. Non adherent cells were washed off and lysates were made from adherent cells. Expression of β-actin was used for normalization.

**PPAR-γ KO mice show reduced protection against tumor challenge after prophylactic GVAX**

Tissue specific deletion of PPAR-γ using LysM-Cre mice allowed us to address the role of myeloid PPAR-γ in GVAX induced anti-tumor immune responses. Mice prophylactically given GVAX (a week before challenge with live WT tumor cells) were protected from tumor growth and showed long term tumor free survival (Fig 10a, b). Tumor growth was not impacted by loss of myeloid PPAR-γ in the absence of prior vaccination (Fig 10b). Surprisingly, we found that vaccine efficacy was reduced in PPAR-γ KO mice (Fig 10b- representative survival curves, statistics on (c), tumor incidence and (d), survival, from four repeats of the study).
Figure 10: Genetic depletion of PPAR-γ in myeloid cells reduces vaccination efficiency in B16 murine melanoma model. A. Schematic of prophylactic vaccine regimen. B. Survival curves of WT and PPAR-γ KO mice. Note that similar results were obtained when “fl only” or “cre only” mice were used as controls. 4 repeats were performed (total vaccinated con n=27, KO n=25). Some KO were protected but KO cohorts always displayed reduced protection against tumor challenge as compared to control C. Tumor incidence on day 60 after tumor challenge. D. Survival on day 60 after tumor challenge (not statistically significant).

These data indicate that PPAR-γ function in LysM-Cre expressing cells is required for full GVAX induced protective immunity. This is an unexpected finding given the immunosuppressive roles that PPAR-γ is known to play. We sought candidate mechanisms to explain the loss of vaccine efficacy in the PPAR-γ KO and found reports that suggested that PPAR-γ function in DC might be important for optimal NKT cell activation. We know from CD1d KO mice that NKT cells are required for GVAX induced tumor protection. In our studies
in CD1d KO mice, loss of NKT cells resulted in the loss of GVAX induced Th2 responses, as measured with splenocytes that were restimulated in vitro with irradiated B16 cells [5]. This GM-CSF/NKT cell/Th2 cytokine axis might be relevant to the loss of vaccination activity in the PPAR-γ deficient mice, as PPAR-γ has been postulated to be important for “M2” activation of macrophages. PPAR-γ KO mice on the Balb/c background are deficient in the Th2 response to Leishmania [6]. Thus, using the restimulation assay as well as other ex-vivo and in vitro assays we tested if NKT function or Th2 generation was impaired in PPAR-γ KO mice.

Restimulation of splenocytes from vaccinated mice does not identify any obvious defect in the PPAR-γ KO anti-tumor cytokine response

Splenocytes harvested a few days after vaccination and cultured with irradiated B16 show a marked cytokine response. Our laboratory has previously shown that the Th2 component of this response is NKT cell mediated. We found that PPAR-γ KO mice generated comparable or slightly enhanced levels of the Th1 and Th2 type cytokines tested, which included IFN-γ, GM-CSF, IL-5, IL-13, and IL-10 (Table 2).
Table 2: PPAR-γ KO splenocytes restimulated with B16 cells do not show an alteration in their cytokine profile. Data are representative of 5-6 mice. 2\times 10^6 splenocytes were cultured with 50,000 irradiated B16 cells. Cytokine levels in the supernatants were measured by ELISA. ND—not detected.

<table>
<thead>
<tr>
<th>Cytokine (ng/ml)</th>
<th>Con Naïve + irB16</th>
<th>KO Naïve + irB16</th>
<th>Con Vax + ir B16</th>
<th>KO Vax + ir B16</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>1.3-8.3</td>
<td>1.7-3.9</td>
<td>3.6-18.3</td>
<td>5.2-18.3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>ND</td>
<td>ND</td>
<td>0.1-1.0</td>
<td>0.3-0.8</td>
</tr>
<tr>
<td>IL-5</td>
<td>ND</td>
<td>ND</td>
<td>0.9-3.7</td>
<td>1.9-6.4</td>
</tr>
<tr>
<td>IL-13</td>
<td>ND</td>
<td>ND</td>
<td>1.8-6.2</td>
<td>3.5-9.9</td>
</tr>
<tr>
<td>IL-10</td>
<td>ND</td>
<td>ND</td>
<td>0.5-6.3</td>
<td>1.5-7.4</td>
</tr>
</tbody>
</table>

**CD1d expression is unaffected in PPAR-γ KO mice**

The PPAR-γ ligand Rosi has been shown to induce CD1d expression in GM-CSF and IL-4 derived human DC. This increased expression leads to an increase in NKT cell activation [7]. Thus we tested the expression of CD1d in PPAR-γ KO myeloid subsets. As shown in Fig. 11, we did not detect a difference in CD1d expression in naïve splenic myeloid subsets defined by either CD11b or CD11c expression. CD11b+ CD11c- cells can either be monocytes, macrophages or neutrophils. CD11b+ CD11c+ cells are considered to be monocyte derived dendritic cells where CD11c+ CD11b- cells are classical DC. Further classification is possible using numerous available markers but we used these two markers as a tool to do a preliminary screen of CD1d expression. We found that in a variety of myeloid cell populations in the naïve spleen, CD1d expression was unaffected in the PPAR-γ KO. As a control, we also tested B-cells, a subpopulation of which (marginal zone B cells) is known to express high levels of CD1d, and found that both wild type and PPAR-γ deficient cells showed comparable expression. However, from studies described in chapter 2 and later confirmed by Gautier et al [8], we knew that PPAR-
γ is not easily detectable in myeloid splenic subset in steady state. We tested splenic myeloid cells in vaccinated mice and again found no defect in the PPAR-γ KO (Fig. 12). As PPAR-γ was robustly detectable in alveolar macrophages, we tested if expression in alveolar macrophages was affected. Even alveolar macrophages from PPAR-γ KO mice had no defect in CD1d expression (Fig. 13).

Figure 11: CD1d expression remains unchanged in naïve PPAR-γ KO spleen. Spleens were mechanically digested and stained for CD11c, CD11c, CD19, CD1d and a dye to discriminate dead cells. Live cells were used to gate on the indicated populations.
Figure 12: CD1d expression remains unchanged in vaccinated PPAR-γ KO spleens. Spleens were mechanically digested and stained for CD11c, CD11c, CD19, CD1d and a dye to discriminate dead cells. Live cells were used to gate on the indicated populations.
Figure 13: Alveolar macrophages from PPAR-γ KO mice retain equivalent surface expression of CD1d. BAL was stained for flow cytometry and alveolar macrophages were identified by CD11c expression and co-labeled with CD1d.

Our studies could not address a defect in CD1d expression in the APC recruited to the vaccine site, as these are technically challenging to harvest and then study by flow cytometry. Thus we used the live-B16 GM vaccine model where continuous release of GM-CSF and a palpable vaccine site allow easy harvest of recruited APC.

Flow cytometry did not reveal a major defect in the PPAR-γ KO APC from live B16-GM vaccination sites

We again used CD11b and CD11c as markers to identify myeloid population in the live-GM vaccine sites (Fig. 14a). In addition, we used Gr-1 to categorize CD11b single positive cells as granulocytic (Gr-1+) or monocytic (Gr-1-) (Fig. 14b). As these vaccine sites are in fact, progressive tumors, it is important to note that their growth was not affected in the PPAR-γ KO mice (Fig 14c). Neither did we did not find any difference in the frequencies of various myeloid
subsets as defined by CD11b, CD11c and Gr-1 in the PPAR-γ KO vaccine sites (14d and e) or their absolute numbers (data not shown). We further tested the activation status of the granulocytic, monocytic and dendritic fraction by using MHCII, CD80, and CD86 and did not find any difference in the PPAR-γ KO (Fig. 15).

Figure 14: A granulocytic, a monocytic and one DC population can be distinguished at the live-GM vaccine site in equal numbers in con and PPAR-γ KO mice. Over 25 control animals and approx. 12 PPAR-γ KO animals were examined. Gr-1 discrimination was conducted on 4 animals, in others CD14 was used to distinguish the monocytic fraction of the CD11b SP.
Figure 15: No difference was detected in activation status of live-GM vaccine site granulocytes, monocytes and DC in PPAR-γ KO. MHCII (left), CD80 (middle) and CD86 (right) staining on DP cells (top two histograms), monocytes (middle two histograms) and granulocytes (bottom two histograms) from con (red) and KO (blue) vaccine sites.

We then tested the CD11b SP (monocytes and granulocytes) and CD11c CD11b DP (monocyte derived dendritic cells) for their expression of CD1d. As shown in Fig. 16, we found no defect in CD1d expression in vaccine site APC from PPAR-γ KO mice. Thus, we concluded that PPAR-γ loss in LysM expressing cells does not affect murine CD1d expression.
Figure 16: CD1d expression on CD11b SP and CD11b CD11c DP cells recruited to vaccine site was not affected in the PPAR-γ KO mice. Vaccine sites were processed on d11-d14. 4-7 animals were processed per group.

We wondered if the published data reporting Rosi effects on cultured human DC could be used to reveal more candidates that might contribute to the reduced vaccine efficacy in the PPAR-γ KO. We reanalyzed publically available datasets and found that PD-L1 expression is reduced by Rosi treatment of cultured human DC (bioinformatics analysis performed by Vladimir Brusic and David Deluca at the DFCI Bioinformatics Core). This suggested that PD-L1 expression could be upregulated in the PPAR-γ KO. PD-L1 is known to be induced by GM-CSF and increased expression on APC could lead to reduction in vaccine efficacy. However, flow cytometric analysis of cells recruited to the vaccine site did not show any defect in PD-L1 (Fig. 17).
Figure 17: PD-L1 expression on myeloid cells recruited to the vaccine site is not affected in the PPAR-γ KO. PD-L1 staining on DP cells (top two histograms), monocytes (middle two histograms) and granulocytes (bottom two histograms) from con (red) and KO (blue) vaccine sites.

The wide range of expression of these activation markers suggested that further subcategorization of these myeloid cells would be possible. We tested the markers CD14, CD103 and Ly6c (Fig. 18). CD14 and Ly6c expression are seen on monocytes. Ly6c expressing cells can be further subdivided into Ly6hi (inflammatory monocytes) and Ly6lo. CD103+ DC are found in several anatomical sites and maintain tolerance via Treg under homeostatic conditions. Yet they are very efficient at cross presentation and mounting a CD8 response during an immune
response [7]. GM-CSF KO have reduced numbers of CD103+ DC in several non-lymphoid compartments. We did not detect a difference in any of these markers or subpopulations in the PPAR-γ KO (data not shown).

**Figure 18: Subsets of APC recruited to the vaccine site.** 6-8 mice were analyzed for con and KO each.
Coculture of vaccine site APC with various effector cell subsets did not reveal any defects in the PPAR-γ KO

Based on expression markers, it appeared that PPAR-γ KO APC from vaccine sites were present and expressed similar surface markers compared to wild type mice. We extended our analysis to investigate the functional capacity of the PPAR-γ deficient APCs. We cultured myeloid cells (using CD11b and CD11c as markers) from B16-GM tumors with CD4 and CD8 cells from the spleens of naive or vaccinated mice. The only T-cells which proliferated in these assays in response to the vaccine site APC were FoxP3+ CD4+ regulatory T-cells from naive mice (Fig. 19a). GM-CSF is known to be required for Treg homeostasis in the gut and can promote Treg in culture. There was no difference in Treg proliferation when the Treg were cultured with vaccine site PPAR-γ KO APC as compared to con APC (Fig 19a). CD4 and CD8 from vaccinated mice produced cytokine in response to the APC but the levels of IL-2, IFN-γ and IL-5 production by CD4 (19b) and IFN-γ production by CD8 (19c) were not different if the APC were derived from PPAR-γ KO mice.
Figure 19: Coculture with naïve or vaccinated CD4 and CD8 live vaccine site APC did not reveal a defect in the PPAR-γ KO. Myeloid cells were collected from B16-GM tumors using magnetic beads and cultured with splenic CD4 and CD8 cells from previously vaccinated or naive mice. A. CFSE dilution of FoxP3+ and FoxP3- CD4 and CD8. B. Cytokine production by CD4. C. Cytokine production by CD8. 50,000 APC were cultured with 500,000 T cells. 7-9 mice were tested per group across 3 experiments.

We also continued our investigation into the role of NKT cells, if any, in the vaccine defect in
the KO. In one study, lipid antigen availability on CD1d was suggested to be modulated by PPAR-γ induced cathepsin D [9]. Thus we cultured NKT with vaccine site APC to measure their cytokine responses. Two different sources of NKT cells were used: cell lines or primary NKT cells derived from Vb7 restricted mice generated by somatic cell nuclear transfer (Stephanie Dougan, unpublished data). Briefly, a nucleus from a Vb7 expressing NKT cell was extracted and placed in an enucleated oocyte which was then allowed to grow to the blastocyst stage. Embryonic stem cell lines derived from the Vb7 blastocysts were injected into WT blastocysts. Chimeric blastocyst were implanted in pseudopregnant mice. The resulting chimeric pups can be mated to obtain Vb7 mouse lines. Since the TCRα locus does not display absolute allelic exclusion in WT animals (30% of all T cells have both alleles of TCRα rearranged and 10% express both alleles), the T cell compartment in extremely restricted but not clonal in these mice. The T-cell compartment in the Vb7 mice is skewed towards NKT cell development though some CD8 T cells are present.

Cytokine profile of a NKT cell line (24.8) or primary Vb7 NKT cells was similar in the presence of APC from con or KO vaccine sites (Fig. 20). The only cytokine detectable on coculture of CD11b+ cells from live-GM vaccine sites and 24.8 cells was IL-2, which was not markedly affected by loading the CD11b cells with α-galactosylceramide (aGC, data not shown). There was no difference in IL-2 production by 24.8 cells when stimulated with KO APC (Fig. 20a). Primary Vb7 NKT cells produced IL-2, IL-5 (Fig. 20b), IL-13 and IFN-γ (Fig. 20c) on aGC stimulation but not with endogenous ligands. Any alteration in CD1d expression or recycling in the PPAR-γ KO APC would impact the NKT cell response to aGC. Similarly if costimulatory ligands, either cell surface or secreted, differ in the KO, it may impact NKT cell response to aGC. However, the cytokine response of primary NKT cells to aGC loaded APC also remained
unchanged when PPAR-γ KO APC were used

**Figure 20:** NKT cells cultured with con or PPAR-γ KO vaccine site APC display similar cytokine profiles. 50000 APC from live-GM vaccine sites were cultured with 50000 24.8 NKT cell clone or Vb7 expressing primary NKT from somatic nuclear transfer mice for 48 hours. For aGC loading, APC were incubated with 500ng/ml aGC for 2-4 hours and then washed repeatedly.

Thus, the studies described here failed to reveal a clear defect in PPAR-γ KO vaccine site APCs as determined from the live-GM vaccine sites.

**DISCUSSION**

PPAR-γ is known to have many immunosuppressive functions in macrophages and dendritic cells. Contrary to our expectation, deletion of PPAR-γ using LysM-Cre reduced the ability of irradiated, GM-CSF secreting B16 cells to stimulate protective immunity against subsequent tumor challenge. Although prior reports suggested a role for PPAR-γ in NKT cell activation, we failed to detect a clear defect involving NKT cells in the PPAR-γ deficient mice. Instead, we
found that a) CD1d expression was unaffected in PPAR-γ KO mice and b) NKT cell activation by vaccine site APC as measured by cytokine release was also unaffected. We also conducted coculture assays with the vaccine site APC with CD4 and CD8 cells from naïve and vaccinated mice but were unable to reveal a defect in the PPAR-γ KO vaccine sites. Moreover, similar myeloid cells were recruited to the site of GM-CSF secreting tumor cells in wild type and PPAR-γ deficient mice. Together, these results raised the possibility that a previously unknown function of PPAR-γ might be involved in the impaired vaccination response, an issue that we address with detailed expression profiling analysis in the next chapter.

REFERENCES


Chapter 4

High throughput analysis of gene expression in GVAX draining lymph node and identification of a novel role of PPAR-γ in myeloid cells

INTRODUCTION

In the introductory chapter, we have reviewed that LysM-Cre; PPAR-γ fl mice recapitulate the autoimmune phenotype of the GM-CSF KO mice. Thus, a reasonable hypothesis would be that GVAX efficacy would be improved in LysM-Cre; PPAR-γ fl mice. However, as discussed in chapter 3, we unexpectedly found a defect in vaccine efficacy in the KO. In the previous chapter, we explored several published candidate mechanisms that might explain this phenotype, but found them to be unaffected in the KO mice. Thus we wanted to explore potentially novel mechanisms in an unbiased fashion. The studies presented here derive from the RNASeq analysis of vaccine draining lymph nodes from control and KO mice. We know from previous work that irradiated, GM-CSF secreting B16 cell vaccines induce both a CTL and antibody response. We hypothesized that if the generation of the protective response was impaired, we might detect specific anti-tumor defects in the lymph node. Additionally, alterations in LN myeloid populations might also be evident.

METHODS

RNASeq

dLN were harvested 5 days after vaccination. LN from 4 mice were pooled and RNA was extracted. RNA was subjected to HiSeq and transcript levels determined for approximately 20,000 genes (Center for Canter Computational Biology, DFCI). 2 technical repeats were
performed for con and 3 for KO.

**Gene Set Enrichment Analysis (GSEA)**

GSEA was performed using all available genesets in the Immgen database (~300 at the time) to identify modules and associated cell types whose gene signature were differentially represented in con or KO LN.

**Combinatorial Immunotherapy**

For the experiments exploring synergy of GVAX+CTLA-4 with Rosi, we used two different challenge doses: $10^5$ or $4 \times 10^5$. Vaccination dose was $3 \times 10^6$ cells B16-GM, injected once, subcu. on the abdomen, opposite to the flank with the challenge dose. Rosi or DMSO were given in drinking water at 20mg/kg/day for 12 days. Mice were injected i.p. with anti-CTLA-4 (9D9, BioXcell) or isotype as follows: 200ug on d0, 100ug on d3 and d6.

**RESULTS**

**Gene expression profiling of vaccine draining lymph node**

In support of the protective response induced by the vaccine, ipsilateral inguinal lymph nodes were dramatically enlarged morphologically and in cellularity (5-10 fold, data not shown). To analyze the vaccine effector mechanisms without bias towards one particular cell type, we collected RNA from draining lymph nodes 5 days after vaccination and conducted RNA-Seq. Draining lymph nodes from 4 mice were pooled to reduce variability.

To identify changes in gene expression in the draining lymph node, the transcript levels obtained
from the RNASeq data were analyzed by gene set enrichment analysis (GSEA). We used the genesets available through Immunological Genome project consortium (Immgen.org) to identify modules corresponding to specific cell types and signaling pathways. Interestingly, a PPAR-γ dependent gene expression module previously shown to be enriched in alveolar macrophages [1] was underrepresented in the PPAR-γ KO lymph node compared to controls, confirming that PPAR-γ dependent myeloid gene expression was reduced (Fig 21a). Genesets that are known to be repressed by PPAR-γ were upregulated in the KO confirming functional deficiency of PPAR-γ (Fig. 21b). Together, these findings indicated that more detailed analysis of the gene expression profiles might provide insights into the impaired vaccine responses in PPAR-γ deficient mice.
**Figure 21: GSEA shows difference in KO dLN consistent with loss of PPAR-γ in myeloid cells.**

dLN were collected 5 days after GVAX and analyzed by RNA-Seq. GSEA was performed to check for enrichment for all modules present in the Immgen database. A. Geneset known be induced by PPAR-γ in myeloid cells. B. Genesets known to be repressed by PPAR-γ.
Interestingly, CTLA4 was one of the top genes showing upregulation in KO lymph nodes (last gene Fig. 21b, previous page). CTLA4 is strongly expressed on regulatory T-cells and on activated and exhausted effector cells. GSEA showed gene expression modules specific to Treg are upregulated in the KO (Fig 22a.). We sought to confirm this possible alteration in Treg by flow cytometry. As shown in Fig 22b, Treg frequency is increased. As Treg are a major regulator of anti-tumor effector T cells, we wondered whether this might impact CD8+ T cells. Indeed, the CD8:Treg ratio was decreased in KO draining lymph nodes compared to control mice 6-8 days after vaccine administration (Fig. 22c).
Figure 22: GSEA and flow cytometry show increased Treg and decreased CD8:FoxP3 ratio in PPAR-γ KO dLN. A. Immgen modules enriched in Treg are shown in red with corresponding p-values for enrichment in KO dLN. B. Representative comparison of con and KO dLN and their CD8:Treg ratio by flow cytometry 6-8 days after vaccination. C. Quantification of LN CD8:Treg ratio. ~25 mice each were evaluated for con and KO mice in 5 experiments.
**CD8:Treg ratio in the tumor is also reduced in the KO**

We wondered if the altered balance of CD8 T effectors and FoxP3+ Treg observed in the draining lymph nodes were also seen at the tumor site. For these experiments, we moved to a therapeutic vaccine model (Chapter 3, introduction) such that all mice would have progressive tumors. As shown in Fig. 23, d14 B16 tumors from GVAX treated KO mice did not show an alteration in the frequency of CD45+ cells in single cell suspensions of tumors. However, the frequency of CD3+ T cells was significantly reduced in the KO mice compared to controls. There was a non-significant trend towards larger tumor sizes. The lack of effect on tumor growth might reflect the limited ability of GM-CSF secreting tumor cell vaccines to impact the progression of established tumors, precluding the ability to detect a major PPAR-γ dependent contribution. Similar results were obtained with d11 tumors (data not shown).

**Figure 23: Analysis of tumor infiltrating leukocytes reveals lower T-cell infiltration in**
**tumors in PPAR-γ KO mice.** Con or KO females were challenged with live B16 cells ($10^5$) and vaccinated with irradiated, GM-CSF secreting B16 cells ($10^6$) at a different site on day one. Tumors were harvested on day 14, weighed, and processed to single cell suspensions, which were then stained with antibodies to CD45 and CD3. Tumor cells were excluded based on size/scatter profiles and lack of CD45 staining. 8-12 mice were studied per group.

We further categorized the CD3 infiltrate based on CD8, CD4 surface expression and intracellular staining for FoxP3. Total recovery of CD8 and Treg was reduced in the KO tumors as expected due to lower CD3 (Figure 24). However, this effect was more pronounced (and statistically significant) in the CD8 compartment, leading to lower CD8:Treg ratios in the KO mice. The balance of CD8 to Treg at the tumor site has emerged as an important prognostic variable for a number of cancers in clinical studies.

![Graphs showing CD8 and Treg percentages and ratios in KO and Con tumors.](image)

**Figure 24: The ratio of CD8+ T cells to FoxP3+ regulatory cells is decreased in tumors**
from vaccinated PPAR-γ KO animals. Con or KO females were challenged with live B16 cells (10^5) and vaccinated with irradiated, GM-CSF secreting B16 cells (10^6) at a different site on day one. Tumors were harvested on day14, weighed, and processed to single cell suspensions, which were then stained with antibodies to CD45 and CD3. Tumor cells were excluded based on size/scatter profiles and lack of CD45 staining. 8-12 mice were studied per group.

**KO LN have increased expression of Treg promoting cytokines CCL17 and CCL22**

To explain the increased Treg frequency and the effect on the CD8:Treg ratio, we returned to our RNASeq data. Interestingly, the expression of chemokines CCL17 (TARC) and CCL22 (MDC) was upregulated in the KO geneset. CCL17 and CCL22 have been implicated in recruiting Treg via their receptor CCR4. Interestingly, the main subset known to produce CCL17 and CCL22 are macrophages and dendritic cells. We tested the changes in CCL17 and CCL22 production by ELISA. Figure 25 shows the increased expression of CCL17 and CCL22 by PPAR-γ KO GVAX dLN at 3 different time points.

![Graphs showing increased expression of CCL17 and CCL22](image)

**Figure 25: KO dLN produce higher levels of Treg attracting chemokines.**
dLN were collected at the indicated time after GVAX. 5X10^5 cells were plated and supernatants collected after 48 hours. Chemokine levels were measured by ELISA. Each data point represents a technical replicate. 3-4 mice were tested per group for each timepoint and sex. A paired comparison was performed on the 5 means (sex and time) for con and KO each to obtain the p-value.

**IFN-γ response of CD8 T-cells is not defective in the KO**

We asked what impact increased Treg had on CD8 function. As shown in Fig. 26, CD8 from con and KO LN secreted equivalent levels of IFN-γ in response to an immunodominant peptide from Trp-2, a melanocyte specific protein that is targeted in the anti-B16 response to GVAX. These data are not surprising as we had already seen equivalent IFN-γ levels in the restimulation of the spleen (chapter 3, Table 2) and the increased IFN-γ response gene signature in the RNASeq (Fig. 21). We are currently optimizing cytotoxicity assays to determine if CD8 mediated killing of B16 tumors is defective as a result of the increased Treg. However, from the RNASeq we did not detect any reduction in granzymes or perforin in the KO (data not shown).
Figure 26: Con and KO CD8 from GVAX dLN produce equivalent levels of IFN-γ in response to Trp-2 peptide. 3-4 LN were pooled and 500,000 lymphocytes plated with 10μg/ml of indicated peptide. Supernatants collected at 48 hours were assayed by ELISA. Data representative of 3 experiments.

**KO LN have an enhanced gene expression signature for Langerhans Cells**

It is possible that PPAR-γ deficiency results in an alteration in the antigen presented in cells in the draining lymph nodes, particularly as myeloid cells are the major producers of CCL17 and CCL22. In this context, an Immgen module for Langerhans’ Cells (LC) was enriched in KO LN compared to controls (Fig. 27). Consistent with this idea, published reports show that PPAR-γ can be expressed by LC.
Figure 27: KO LN have increased expression of a Langerhans Cell specific gene module.

dLN were collected 5 days after GVAX and analyzed by RNA-Seq. GSEA was performed to check for enrichment for all modules present in the Immgen database.
We used the Immgen database to check if Lysozyme M is expressed in LC and thus could be directly impacted by the PPAR-\(\gamma\) deletion. As shown in Fig. 28, LC did express Lysozyme M.

LC travel to the cutaneous lymph nodes upon activation. LC express langerin or CD207. However in recent studies dermal DC have also been shown to express CD207. Further discrimination based on EpCAM and CD103 is possible though there is still some debate over their utility in defining skin dendritic cell subsets [2]. Fig. 29 shows our staining strategy to identify LC and discriminate between LC and dermal langerin expressing DC. We identified LC as CD207+ EpCAM+ cells. We could detect two subsets based on CD103 expression. Further we could detect a CD207- MHCIIhi EpCAM- dendritic cell subtype. All 3 subsets of DC expressed CCR7, suggesting that these are migratory DC. Thus, the CD207- subset might be dermal DC. As expected the LC were negative for CD8 expression.
Figure 28: LC express modest levels of lysozyme M. The Gene Skyline data viewer in Immgen was used to visualize Lysozyme M expression in key leukocyte populations.
Figure 29: Staining strategy for Langerin expressing DC in the lymph node. Lymph nodes were mechanically digested to obtain single cell suspensions. Gated on live B220- MHCIIhi cells.

Based on our gating strategy we could not identify a numeric defect in total LC or the relative population of CD103+ LC (Fig. 30). Further studies are required to determine if LC function is
altered with PPAR-\(\gamma\) deficiency. We are planning coculture studies of LN APC with various T cell subsets to identify potential functional defects in the KO.

![Graph showing CD207+ cells and CD103 expression in KO and con mice.](image)

**Figure 30:** Total CD207+ cells or the frequency of CD103 expression is unaffected in the PPAR-\(\gamma\) KO. At least 14 mice each were analyzed for con and KO LC across 4 experiments.

**Pharmacological activation of PPAR-\(\gamma\) using Rosiglitazone showed consistent gain-of-function phenotypes and identified Rosiglitazone as an immunotherapeutic**

Several synthetic agonists of PPAR-\(\gamma\) are available. One of these, Rosiglitazone (Rosi), is well characterized and is clinically approved for the management of diabetes. Given that in our model system, selective loss of PPAR-\(\gamma\) causes increased Treg numbers, we tested if Rosiglitazone treatment in mice treated with GVAX would reduce Treg numbers and improve the CD8:Treg ratio in the LN and the tumor.

Rosi is given orally to patients. Therefore, we decided to deliver it via drinking water to mice. We started Rosi treatment on the same day as vaccination. To make the Rosi GOF experiments
comparable to the genetic LOF, we compared DMSO and Rosi treated LN 6-8 days after vaccination. As shown in Fig. 31, there were no significant differences in CD8 or Treg frequency or in the CD8:Treg ratio in Rosi or DMSO treated GVAX mice (there appears to be a trend towards an increased CD8/Treg ratio).

![Figure 31: Rosi does not impact the balance between CD8 and Treg in the vaccine draining lymph node after 6-8 days of treatment.](image)

Data representative of 3 experiments with 4-5 mice per group.

However, Rosi treatment for 12 days showed significant enhancement on the tumor infiltrating lymphocytes (Fig 32). Strikingly, while KO mice had reduced CD3 infiltration, Rosi treated mice had improved CD3 infiltration and total CD45+ infiltration. Consistent with this, while absolute numbers of CD8 and Treg were higher, Rosi treated mice had higher CD8:Treg ratio. This gain-of-function phenotype is consistent with the genetic loss-of-function of PPAR-γ in the myeloid lineage.
Figure 32: 20mg/kg/day Rosi delivered via drinking water improves the intratumoral CD8:Treg ratio in GVAX treated mice. Mice were challenged with 10^5 live tumor cells (left flank) and vaccinated with 10^6 irradiated B16-GM cells (abdomen). Rosi or DMSO were added to their drinking water for 12 days. Tumors were harvested on day 14. Data pooled from 2 experiments. Each data point represents one mouse.

Improved CD8:Treg ratio with systemic delivery of Rosi requires myeloid PPAR-γ

It is expected that oral Rosi treatment would impact several cell types. Thus we wanted to address if Rosi mediated improvement in immune infiltrates did require myeloid PPAR-γ. As shown in Fig. 33, CD45+ infiltrate, CD3+ infiltrate as well as CD8:Treg ratio remained unchanged (no statistical significance) with Rosi treatment in the absence of PPAR-γ expression in myeloid cells.
Figure 33: Rosi mediated improvement in immune infiltrate requires PPAR-γ expression in myeloid cells. Mice were challenged with $10^5$ live tumor cells (left flank) and vaccinated with $10^6$ irradiated B16-GM cells (abdomen). Rosi or DMSO were added to their drinking water for 12 days. Tumors were harvested on day 14. Data pooled from 2 experiments. Each data point represents one mouse.
Rosi improves the anti-tumor response to combinatorial treatment with GVAX and CTLA-4 blockade

We noticed that Rosi treatment of mice vaccinated with irradiated, GM-CSF secreting B16 cells did not impact the size of the challenge tumor despite having an improved CD8:Treg ration (Fig. 32). Consistent with this result, the combined treatment failed to prolong survival (data not shown). We wondered, however, whether the improvement in the CD8/Treg ratio might result in enhanced efficacy of other combinatorial strategies known to augment vaccination potency. In this context, CTLA-4 antibody blockade is known to improve intratumoral CD8 function and to deplete intra-tumoral Treg in combination with GVAX. CTLA-4 had also emerged as an upregulated gene in KO dLN. Further, T-cells (both effector and regulatory) homing to B16 are known to express CTLA-4. Thus, we tested the effect of Rosi treatment on the response to GVAX+anti-CTLA4. As shown in Fig 34, Rosi treatment significantly increased survival with GVAX+anti-CTLA4. The benefits of Rosi were observed against two different challenge doses.
Figure 34: Rosi potentiates the efficacy of GVAX+anti-CTLA-4 treatment. As described in methods, mice received challenge and vaccination (3X10^6) on the same day. Rosi treatment was given for 12-14 days via drinking water (20mg/kg/day). Anti-CTLA4 or isotype were injected i.p. on d0 (200ug), d3 (100ug) and d6 (100ug).
DISCUSSION

We have revealed a previously unidentified function of PPAR-γ in myeloid cells: restraining Treg numbers in response to GM-CSF secreting tumor cell vaccination in mice. This function of PPAR-γ differs from the previously described immunosuppressive effects. However, in our assays, this immunostimulatory role of PPAR-γ is dominant, as GVAX efficacy is reduced in the KO. We have demonstrated that PPAR-γ loss resulted in increased Treg numbers in dLN and tumors, decreased effector to Treg ratios and increased Treg recruiting cytokines in lymph node supernatants. To delineate the contribution of increased Treg to the vaccine defect, we are testing vaccine efficacy in con and KO mice depleted of preexisting Treg using an anti-CD25 antibody.

We were able to demonstrate consistent GOF phenotypes using a synthetic ligand of PPAR-γ, Rosiglitazone. Further, we were able to show that Rosi can potentiate the immune response to GVAX+anti-CTLA-4. These are clinically relevant data as Rosi is an FDA approved small molecule and could be evaluated in patients as a potential immunotherapeutic. Thus, in chapter 5, we investigate the effect of Rosi in an assay with freshly isolated human PBMC where the effects of GM-CSF in stimulating Treg in humans can be studied.

REFERENCES


Chapter 5

Effect of PPAR-γ modulation in studies of GM-CSF function in human PBMC

INTRODUCTION

GM-CSF derived dendritic cells in mice and humans are known to be tolerogenic in the absence of any costimulation. Various coinhibitory ligands (PDL1, Ox40L) have been implicated in these assays. Nicholas Souders in our laboratory found that culturing human PBMC with a GM-CSF expressing cell line (K562, erythroleukemia cells) for 4 days can lead to a selective increase in the number and frequency of Treg (unpublished data, repeated in Fig. 35b). Karrie Wong further extended these data by studying the impact of GM-CSF on the adherent population in these assays. Adherent cells from human PBMC are largely monocytes. Not surprisingly, GM-CSF treatment led to increased number of monocytes. However, GM-CSF exposed monocytes expressed reduced levels of HLA-DR (unpublished data, repeated in Fig. 37). Using these assays, we tested the effect of PPAR-γ activation and inactivation with small molecules on the phenotype of GM-CSF exposed human PBMC.

METHODS

Culture of human PBMC with GM-CSF

Human PBMC were obtained by gradient centrifugation of leukapheresis collars from platelet donors. 4X10^6 cells were plated with 10^5 K562-WT or K562-GM. Control and GM treated conditions were exposed to 10uM Rosi or DMSO every 48 hours. On day 4-6 of culture, cells were harvested. Adherent cells were obtained by incubation with 2mM EDTA at 37°C. Cells
were stained for flow cytometry in the presence of 1mM EDTA. Dead cells were discriminated by using the Live/Dead Fixable dyes from Invitrogen. Antibodies were sourced from BD Biosciences, Biolegend and Ebioscience.

**PPAR-γ modulation**

Rosi was obtained from Adipogen as a powder. It was resuspended in DMSO and 10uM Rosi or equal volume of DMSO was used every 48 hours. T0070907, an antagonist of PPAR-γ [1], was used at 1uM added every 48 hours.

**CCL17 measurement**

CCL17 levels were measured using ELISA (DY364, R&D Systems).

**RESULTS**

*Human peripheral blood mononuclear cell cultures show increased Treg cells on treatment with GM-CSF which is counteracted by myeloid PPAR-γ agonism*

We found that PPAR-γ ligand Rosi can reduce the extent of GM-CSF induced Treg expansion (Fig. 35a, b). The conservation of this pathway between mice and humans is further emphasized by the increase in GM-CSF induced Treg expansion by PPAR-γ antagonist (Fig. 35c). The studies with PPAR-γ antagonist mimic the murine genetic loss-of-function. PPAR-γ modulation was only effective in the presence of GM-CSF and not in cultures with K562-WT.
Rosi reduces CCL17 production by primary human monocytes treated with GM-CSF

To test if PPAR-γ activation would also reduce the chemokine overexpression that was seen in the murine loss-of-function studies, we cultured CD14+ cells from human PBMC with GM-CSF. In addition, these cultures were treated with DMSO or Rosi. In preliminary data, we find that Rosi treatment reduces CCL17 production by human monocytes treated with GM-CSF (Fig. 36).
Figure 35: Treatment of human PBMC with GM-CSF and PPAR-γ modulators recapitulates Treg effects seen in murine studies. A. Two representative donor PBMC treated with Rosi. B. Treg numbers quantified for each donor. Each data point represents one donor. C. Effect of PPAR-γ inhibition on Treg numbers in human PBMC cultures treated with GM-CSF.
Figure 36: CCL17 expression by GM-CSF treated human monocytes is reduced upon Rosi treatment. $10^6$ CD14+ human PBMC were cultured for 5 days with GM-CSF with 10uM Rosi or vehicle control and CCL17 was measured by ELISA. CCL17 levels were normalized to the number of monocytes per well. Number of monocytes did not differ between con and Rosi treated wells.

Rosi did not impact activation status of control or GM treated monocytes in the adherent PBMC

We next evaluated the impact of Rosi treatment on the myeloid cells in culture. Total myeloid cells were calculated based on scatter and CD14 positivity. HLA-DR and CD40 expression was quantified as a measure of activation. Further the adherent cells expressed CD1c suggestive of a dendritic cell phenotype (data not shown). Total myeloid cell number, activation status or expression of CD1c was not affected in Rosi treated control or GM conditions (Fig 37).
DISCUSSION

The studies described above show the role of PPAR-\(\gamma\) in restraining GM-CSF induced Treg is conserved in humans. In the PBMC culture, all cells are exposed to Rosi and thus it is possible that we are observing the sum of effects on various cell types. However, it is important to note that Rosi is able to reduce Treg number only in the presence of GM-CSF implying a requirement
for myeloid cells. Together with the murine data, our studies have identified a novel and therapeutically important function of PPAR-\(\gamma\). We discuss the collective impact of our studies and potential clinically utility of Rosi in the following chapter.

REFERENCES

Chapter 6

Discussion

In the studies presented in this dissertation, we unexpectedly found a defect in vaccine efficacy and immune correlates in LysM-Cre; PPAR-γ fl mice treated with GVAX. Published literature on PPAR-γ suggested that PPAR-γ expression in myeloid cells is immunosuppressive with rare exceptions. We explored the potential effect of PPAR-γ deficiency on NKT cells and on PDL1 expression but were unable to reveal a defect in these pathways. Thus, we turned to an unbiased high-throughput approach to identify novel pathways modulated by myeloid PPAR-γ.

RNASeq identified a holistic defect in Treg numbers and recruitment in the KO vaccine dLN. We found that these defects were also maintained at the tumor site. As a result of these defects, the balance between effector to regulatory T cells at the tumor becomes inefficient at controlling tumor growth.

PHENOTYPE AND FUNCTION OF INTRATUMORAL TREG

Studies as early as 1989 [1] found lymphocyte infiltration to be predictive of metastases and survival in melanoma. However, it was not until 1995 that “suppressor” T-cells were re-identified, using CD25 as a marker [2]. FoxP3 was identified as the master regulator of Treg differentiation only in 2003 [3-5]. Till this time, intratumoral T-cell presence detected using anti-CD3 antibodies was considered and found to be lacking as a prognostic indicator [6]. Lloyd Old’s group took advantage of Treg markers to study TILS in ovarian cancer patients and showed that CD8:Treg ratio provided the best stratification of overall survival, when compared to any cell type alone or total CD3+ T-cells [7]. Several studies have since found that the balance between intratumoral effector and regulatory T cells [8] correlates with survival (positively in
most cancers; negatively in cancers hypothesized to be inflammation driven cancers such as colorectal cancer).

Increased recruitment (for example, recruitment mediated by CCL17 and CCL22) is one cause of intratumoral Treg accumulation. All of the recruitment factors identified thus far can also recruit other types of T-cells in different contexts. How the tumor uses these broad recruitment factors to preferentially recruit Treg is unknown. Naïve T cells express Foxp3 and convert to a regulatory phenotype upon receiving a strong antigenic stimulus and IL-10 and TGF-β- induced signals, which are both abundant in the tumor microenvironment. Another important factor that affects Treg numbers in the tumor is the expression of the enzyme IDO, which diverts T-cell differentiation into a regulatory phenotype at the cost of a Th17 phenotype. It can also support Treg expansion in autologous cultures with human monocyte derived dendritic cells.

Intratumoral Treg are known to have an “effector-memory” phenotype suggesting that they are antigen experienced. They express an abundance of cell surface inhibitory molecules such as CTLA-4, PD-1, CD39 and CD73 etc. Intratumoral Treg can suppress effector T cell and DC function using well characterized pathways: production of soluble modulators such as TGF-b or contact dependent inhibition using CTLA-4 etc. Other, not so common, tumor restricted functions of Treg include induction of apoptosis in effector T-cells using granzymes, production of adenosine using ectonucleotidases expressed on their surface and production of prostaglandin E2. Tumor infiltrating Treg also have non-immune effects such as induction of angiogenesis via VEGF.
INCREASED EXPRESSION OF TREG RECRUITING CYTOKINES IN PPAR-γ KO MICE

We found that PPAR-γ KO LN had higher expression of CCL17 and CCL22. Table 3 taken from a review [9] on Treg trafficking shows CCL17 and CCL22 have been frequently implicated in recruiting Treg. However, their relative effects on recruiting various T cell subsets are context dependent. CCL17 for instance, is known to reduce rather than recruit Treg in atherosclerosis [10]. It has also been linked to an improved Th2 response [11]. Based on RNASeq, CCR4 (the receptor for CCL17 and CCL22) was actually downregulated in the whole LN. The relevance of this finding is unclear particularly as we were unable to detect CCR4 by flow cytometry (data not shown).

CCL17 has independently been detected as a GM-CSF and PPAR-γ dependent gene in an expression analysis [12, 13]. Most ex-vivo studies of CCL17 function are conducted on GM-CSF derived dendritic cells. Interestingly, CCL17 was found to be an indicator of better prognosis in a tumor vaccine study where the patients were administered GM-CSF in addition to a peptide vaccine. However this effect was only seen in patients treated with cyclophosphamide, a Treg modulation agent [14]. Given CCL17’s apparently conflicting effects on helper T-cells as well as regulatory T cells, one hypothesis to reconcile the above data would be that CCL17 has immunostimulatory functions in addition to induction of Treg; and the former dominate once Treg are suppressed. Our laboratory has previously described a GM-CSF dependent upregulation of CCL22 and induction of Treg from dendritic cells treated with apoptotic thymocytes [15]. Thus, it is not surprising that CCL22 mediated Treg induction should play a role in the vaccine response induced by GM-CSF dependent cellular vaccine. To our knowledge, CCL22 has not previously been linked to PPAR-γ.
CCL17 secretion, so far, has only been seen in myeloid cells. The producers of CCL22 also, most often, appear to be of myeloid origin. However in rare studies, CCL22 has been shown to be expressed by CD8 cells and NK cells. We have confirmed CCL17 and CCL22 overexpression in the KO dLN by ELISA. In preliminary data, we found that Rosi reduces CCL17 expression in

Table 3: Chemokines implicated in Treg recruitment

<table>
<thead>
<tr>
<th>Chemokine/Integrin</th>
<th>Receptor</th>
<th>Context</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL19, CCL22</td>
<td>CCR4</td>
<td>Thymus, HC</td>
<td>Human</td>
</tr>
<tr>
<td>CCL1</td>
<td>CCR8</td>
<td>Thymus, HC</td>
<td>Human</td>
</tr>
<tr>
<td>CCL19</td>
<td>CCR7</td>
<td>Secondary LN</td>
<td>Human</td>
</tr>
<tr>
<td>CD62L</td>
<td></td>
<td>Secondary LN</td>
<td>Mouse</td>
</tr>
<tr>
<td></td>
<td>CCR2</td>
<td>Secondary LN</td>
<td>Mouse</td>
</tr>
<tr>
<td></td>
<td>CCR5</td>
<td>Secondary LN</td>
<td>Mouse</td>
</tr>
<tr>
<td>CXCL13</td>
<td>CXCR5</td>
<td>B-cell Follicles</td>
<td>Human</td>
</tr>
<tr>
<td>CCL4</td>
<td>CCR5</td>
<td><em>Paracoccidioides brasiliensis</em></td>
<td>Human, mouse</td>
</tr>
<tr>
<td>αE (CD103)</td>
<td>E-cadherin</td>
<td><em>Leishmania major</em></td>
<td>Mouse</td>
</tr>
<tr>
<td>CCL28</td>
<td>CCR10</td>
<td>Liver</td>
<td>Human</td>
</tr>
<tr>
<td>CXCL12</td>
<td>CXCR4</td>
<td>Bone marrow</td>
<td>Mouse</td>
</tr>
<tr>
<td>CCL22</td>
<td>CCR4</td>
<td>Ovarian cancer</td>
<td>Human</td>
</tr>
<tr>
<td>CCL17, CCL22</td>
<td>CCR4</td>
<td>Hodgkin's lymphoma</td>
<td>Human</td>
</tr>
<tr>
<td>CCL17, CCL22</td>
<td>CCR4</td>
<td>Non-Hodgkin's lymphoma</td>
<td>Human</td>
</tr>
<tr>
<td>CCL17, CCL22</td>
<td>CCR4</td>
<td>Gastric cancer</td>
<td>Human</td>
</tr>
<tr>
<td>CCL17, CCL22</td>
<td>CCR4</td>
<td>Esophageal squamous cell carcinoma</td>
<td>Human</td>
</tr>
<tr>
<td>CCL22</td>
<td>CCR4</td>
<td>Breast cancer</td>
<td>Human</td>
</tr>
<tr>
<td>CCL17</td>
<td>CCR4</td>
<td>Breast cancer</td>
<td>Mouse</td>
</tr>
<tr>
<td>CCL22</td>
<td>CCR4</td>
<td>Lewis lung carcinoma</td>
<td>Mouse</td>
</tr>
<tr>
<td>CCL2</td>
<td>CCR4</td>
<td>Glioma</td>
<td>Human</td>
</tr>
<tr>
<td>CCL3, CCL4, CCL5</td>
<td>CCR5</td>
<td>Pancreatic adenocarcinoma</td>
<td>Mouse</td>
</tr>
<tr>
<td>CCL20</td>
<td>CCR6</td>
<td>Epstein-Barr virus + Hodgkin's lymphoma</td>
<td>Human</td>
</tr>
</tbody>
</table>
human monocytes cultured with GM-CSF showing that the PPAR-γ mediated downregulation of CCL17 is conserved. However CCL17 has not been reported to impact Treg survival or proliferation. The human PBMC assay where we see decreased Treg numbers argues for an independent effect of Rosi on Treg numbers in addition to CCL17 mediated recruitment.

**Potential mechanisms underlying the upregulation of CCL17 and CCL22 in KO mice**

1. As discussed above, there is limited evidence for a genetic link between PPAR-γ and CCL17 or CCL22. However, both CCL17 and CCL22 have numerous NF-κB binding sites in their genomic loci (Consite.org). The only mechanism known by which PPAR-γ can directly suppress expression is through transrepression of NF-κB mediated transcription. Binding of sumoylated PPAR-γ to NF-kB corepressors prevents their ubiquitination and degradation.

2. An increase in CCL17 and CCL22 may also be an indirect effect of increased or dysregulated frequency of a PPAR-γ dependent myeloid subset. We have not found any flow cytometric evidence for upregulated myeloid populations in our analyses yet the RNASeq data suggest that LC numbers or function may be enhanced. CCL17 and CCL22 are known to be expressed by LC and have important roles in their migration and function [16-18].

**IDENTITY OF THE DEFECTIVE MYELOID CELL POPULATIONS**

We have shown that depletion of PPAR-γ using the LysM-Cre in the myeloid lineage attenuates the response to GVAX. However we do not know the identity of the myeloid cells causing this defect. We were not able to detect any defects in vaccine site myeloid cells using live B16-GM. We are currently confirming by qPCR if myeloid cells recruited to live B16-GM sites express PPAR-γ. Histological analysis of irradiated B16-GM vaccine sites have shown subtle defects which would need extensive analysis and may or may not impact vaccine efficacy.
The loss of the PPAR-γ myeloid signature module in KO LN suggests that the defect lies at the dLN. This module is macrophage specific and is overrepresented in alveolar macrophages [19] where PPAR-γ plays an important role. One possible myeloid candidate to emerge from the RNaseq is the skin resident DC, Langerhans cells (LC). Despite the lack of difference in frequency or numbers of LC by flow cytometry in KO dLN, it is quite possible that LC function is affected.

MODEST KO PHENOTYPE IDENTIFIES A THERAPEUTICALLY IMPORTANT PATHWAY

The defect in vaccine response and the corresponding increase in Treg and Treg recruiting cytokines are variable and modest in PPAR-γ KO mice. It is important to note that PPAR-γ has known immunosuppressive effects, genesets for which were reduced in the KO. Hence, the modest defects that we see are the sum of immunosuppressive and pro-tumor immunity effects of PPAR-γ. This might explain why our studies on vaccine site myeloid cells did not reveal any defects. Further, loss-of-function effects in the KO were consistent with murine and human gain-of-function studies.

The biology that we have revealed is therapeutically important. PPAR-γ activation using the FDA approved synthetic ligand Rosi, improves the response to GVAX+anti-CTLA-4 in our mouse model. These findings are exciting because, anti-CTLA-4 (Ipilimumab) is a recently approved immunotherapy. Sequentially delivery of GVAX and anti-CTLA4 has been tested in patients and our data suggests that a triple combination of Rosi, GVAX and anti-CTLA-4 could achieve significant benefits. Therapies where published data provides a rationale to test a synergy with Rosi are discussed in “Future Preclinical Studies”.
With GVAX alone, Rosi improved CD8:Treg ratio but had no impact on tumor size and survival. Several studies have shown that while treatment with a single antibody against coinhibitory molecules is insufficient, double blockade or Treg depletion can lead to successful regression in murine tumor models. There are also concerns that checkpoint blockade in the presence of Treg might activate the Treg in addition to activating the effector cells though this does not appear to be true of anti-CTLA4 treatment [20].

A small molecule drug to reduce intratumoral Treg

In mice anti-CD25 depletion synergizes with CTLA-4 blockade despite its inefficacy in depleting intratumoral Treg as a monotherapy [21]. With anti-CD25 monotherapy, the effector T-cells generated peripherally are unable to enter the tumor [22]. Moreover, this strategy is impractical for clinical use as it could affect the CD25 levels on effector T-cells [23, 24]. Rosi provides a clinically approved small molecule therapy to target intratumoral Treg.

FUTURE PRECLINICAL STUDIES

Some promising avenues of future preclinical work are discussed below.

1. The myeloid restricted expression pattern of CCL17 and CCL22 gives us an opportunity to identify the myeloid population causing the defect in the KO using fractionation studies in combination with chemokine detection.

2. Our laboratory is also testing a nanomaterial based approach to GVAX. Porous scaffolds are loaded with tumor antigen and GM-CSF and implanted subcutaneously. While data are limited, it is believed that greater temporal and spatial control of GM-CSF release (as well as antigen and adjuvants) can be achieved using this strategy. Moreover, higher local doses of therapeutics can be achieved with low or absent systemic concentrations.
Systemic Rosi treatment can impact several different cell types, both immune and non-immune. In particular, PPAR-\(\gamma\) expression in fat resident Treg is necessary for their maintenance and function [25]. However, it is not clear if pharmacological activation of PPAR-\(\gamma\) would be sufficient to increase fat resident Treg and if this increase would impact the immune response to GVAX. In one study, PPAR-\(\gamma\) has also been reported to suppress Th17 differentiation [26]. We are testing if local delivery of Rosi and GVAX at the vaccine site and draining lymph node using porous scaffolds will result in better synergy between the two.

3. We are currently testing if we observe similar roles for PPAR-\(\gamma\) in GVAX induced immunity in other tumor models. In the spectrum of lymphocyte recruitment in patient cancers, melanoma appears to be one of the more infiltrated tumors. It would be interesting to test if basal infiltration of tumor affects the therapeutic ability of Rosi. Moreover, Rosi can be directly cytotoxic to some murine and human cell lines. It is essential to investigate if direct cytotoxic effects of Rosi can synergize with its effect on the CD8:Treg ratio.

4. Testing KO mice and Rosi treatment with other vaccination approaches, both cellular and non-cellular will allow further elucidation of the immunostimulatory roles of PPAR-\(\gamma\) and its therapeutic value. One obvious synergy that could be explored is with blockade of the PD-1/PD-L1 pathway coupled with Rosi treatment. We know that PD-1 is expressed in TILS in GVAX treated mice, yet PD-1 did not emerge as a differentially expressed gene in the RNAsSeq of KO dLN. Combination with PD-1 blockade will inform our understanding of the mechanisms of synergy between Rosi and checkpoint blockade. A coinhibitory receptor that is elevated in the KO dLN is the newly identified TIGIT. In
preliminary data, we have seen that all Treg from GVAX dLN or tumor sites (from con or KO animals) are TIGIT positive. A recent study has provided proof-of-principle that TIGIT blockade can be immunotherapeutic [27]. Given our data on Treg in GVAX and role of PPAR-γ, the triple combination of GVAX, TIGIT blockade and Rosi appears to be a promising avenue to explore.

5. Most of the studies conducted to evaluate synergy of PPAR-γ activation with chemotherapy were conducted in xenograft models. Remarkably, the few studies done on chemically induced or genetic models of cancer did not look at the host immune system [28]. Our studies raise the exciting possibility that the improvement of the host immune response is contributing to the synergy. Another chemotherapy originally designed to be cytotoxic but later found to have immune effects is gemcitabine which causes apoptosis of myeloid derived suppressor cells (MDSC) and a change in their cytokine profile [16]. There are preliminary data from one study showing that gemcitabine and Rosi can cooperate to reduce MDSC and Treg number [29].

CLINICAL UTILITY OF ROSIGLITAZONE IN CANCER IMMUNOTHERAPY

Rosi (Avandia) and pioglitazone (Pio, Actos) are the two ligands of PPAR-γ that are approved for use in diabetics. Clinical use of Rosi has been fraught with controversy in the past decade. Based on a 2007 meta-analysis which indicated increased cardiotoxicity compared to other glucose lowering medications, the FDA mandated a restricted access program. However, a further clinical trial by Glaxo-Smith Kline and the subsequent FDA review in 2013 lead to the restrictions being removed. It would be fair to say that the controversy is still going on as experts argue which set of studies to accept, the significance of the numbers of patients and cardiotoxic events etc. Pio has not been suspected of increasing cardiotoxicity. There is no preclinical or
clinical data to indicate the physiological reasons for the difference in potential cardiotoxicity as Rosi and Pio are structurally very similar. However, Pio has recently come under scrutiny due to possible increase in the risk of bladder cancer.

Both the cardiotoxicity and bladder cancer, if proved to be real side effects, are rare events. The toxicity of Rosi and Pio is far better than what is considered acceptable for chemotherapeutics.

Yet, we are considering other strategies to mitigate systemic effects especially immunosuppressive downstream of PPAR-\(\gamma\) ligation. In “future preclinical studies”, we have described one such strategy, the use of polymer based scaffolds to deliver GVAX and Rosi.

Short term delivery, following GVAX administration is another strategy which is known to be absolutely safe in patients.

REFERENCES


APPENDIX I

Publications
Discussions on publications of interest

Published online in

“Highlights of recent literature”

Translational Immunology Update

A quarterly publication of the Federation of Clinical Immunology Societies (FOCIS)

Reviewed by Girija Goyal and Glenn Dranoff
Exhausted T Cells in the Tumors but Not the Blood


This fascinating study is the first to compare systematically the gene expression profiles of tumor-specific circulating and intra-tumoral CD8+ T cells. The authors demonstrate that tumor infiltrating CD8+ T cells manifest an exhausted phenotype, whereas circulating T cells bear resemblance to anti-viral effector T cells. These data substantially extend prior work that had shown the upregulation of inhibitory receptors on tumor infiltrating CD8+ T cells.

The authors isolated melanoma specific CD8+ T cells from patients vaccinated with Melan-A/MART-1 derived peptides admixed with CpG, a relatively potent immunization strategy. Specific tetramers were used to purify CD8+ T cells from both blood and lymph node metastases.

- The authors optimized conditions for expression profiling of small numbers of specific CD8+ T cells. Their analysis of circulating naive and viral specific (CMV or EBV) CD8+ T cells was consistent with prior studies, validating their techniques.

- The authors then determined the expression profiles of Melan-A/MART-1 specific CD8+ T cells harvested from the blood or tumors. A comparison of these profiles with published signatures of different stages of T cell effector differentiation, memory, and exhaustion revealed that the circulating tumor-specific T-cells are late effector cells, whereas the tumor infiltrating T cells are exhausted cells.
• The authors confirm the cell surface expression of multiple inhibitory receptors such as Tim-3, PD-1, and CTLA-4 on exhausted, tumor infiltrating CD8+ T cells.

These data highlight key differences between circulating and tumor infiltrating CD8+ T cells. The findings suggest that there may be important limitations to the analysis of peripheral T cells as an index of anti-tumor immunity. Moreover, the demonstration that exhausted, tumor infiltrating T cells express multiple inhibitory receptors raises the possibility that combinatorial therapies may be required to re-invigorate tumor immunity.

VGEF-Secreting Treg link Tumor Hypoxia and Angiogenesis


In this important paper, Coukos and colleagues define a novel pathway that links tumor hypoxia, Treg recruitment, and angiogenesis. The authors show that tumor hypoxia stimulates CCL28 production, which attracts VEGF secreting Tregs that promote tumor angiogenesis.

• Through screening a panel of ovarian cancer cell lines, the authors found that hypoxia selectively enhanced CCL28 production, among a large number of chemokines examined.
• A correlation was established in ovarian cancer patients between shorter survival times and greater intra-tumoral CCL28 levels.
• In a murine ovarian cancer model, the authors showed that enforced expression of CCL28 enhanced tumor growth, and this was associated with increased numbers of intra-tumoral Tregs.
• CCL28 signals through CCR3 and CCR10. The authors found that Tregs express CCR10, while CD8 effector T cells express both CCR3 and CCR10. Depletion of CCR10+ cells, but not CCR3+ cells significantly reduced tumor growth.

• High levels of VEGF and robust angiogenesis were linked with CCL28 overexpressing tumors and CCR10+ Tregs. Moreover, human Tregs produce VEGF under normoxic conditions, but this level may be increased with hypoxia.

These data provide new insights into how tolerance, hypoxia, and angiogenesis are interconnected. The inhibition of tumor growth in response to depletion of CCR10 positive cells suggests that this receptor may be an attractive target for cancer immunotherapy.
A Tumor Tyrosine Kinase Inhibitor Does More Than it was Designed For


Imatinib is a tyrosine kinase inhibitor that blocks the activity of mutant c-kit in gastrointestinal stromal tumors (GIST). Balachandran et al reveal an unexpected role of imatinib treatment in reducing immunosuppression caused by high Ido expression in GIST. Ido (indoleamine 2,3-deoxygenase) converts tryptophan into metabolites that promote the survival of regulatory T cells. Tryptophan depletion concomitantly causes cell cycle arrest in effector T cells.

Imatinib-treated GIST patients show marked improvement in survival but eventually progress due to secondary mutations in KIT. The authors were interested in the immune response to GIST and to imatinib therapy.

- In a murine model of GIST, resected tumors show significant T reg and CD8T cell infiltrate. Imatinib treatment increased CD8⁺T cells frequency and decreased T reg frequency. Cell depletion by anti-CD8 antibody showed that CD8⁺T cells are required for complete imatinib efficacy.

- To understand how imatinib enhanced CD8⁺T cell function, the authors performed a gene array of mouse GIST tumors and found Ido expression dramatically reduced in imatinib treated GIST.
• Ido inhibition caused T reg apoptosis and a CD8\(^+\)T cell-dependent reduction in tumor burden. Treatment with Ido metabolites reduced imatinib efficacy. These data suggest that imatinib reduces Ido expression and enhances anti-tumor function of CD8\(^+\)T cells.

• Reduced Ido expression by imatinib was mediated through c-KIT as seen in a comparison of isogenic cell lines expressing imatinib sensitive vs. resistant c-KIT. c-KIT may mediate Ido induction through mTOR and transcription factor Etv4.

• In GIST patients, an increased CD8 to T reg ratio was seen in resected tumors that had responded to imatinib. Untreated and imatinib resistant tumors showed a much lower CD8\(^+\)T cell: T reg ratio.

This study provides evidence that therapies targeted to tumors cells interact with the immune system in unexpected ways. Based on this study, combinatorial treatment with imatinib and Ido inhibitors appears to be a promising strategy to reduce the development of imatinib resistance.

Lacking IDO, Some Tumors Find Other Ways to Suppress Immunity


Opitz, Litzenburger et al. extend the importance of tryptophan catabolism in anti-tumor immunity by showing that IDO-negative tumors upregulate the related enzyme tryptophan 2,3-dioxygenase (TDO) to convert tryptophan (Trp) to kynurenine (Kyn). Consistent with previous murine studies, they show that Kyn promotes tumor progression and immunosuppresssion through the aryl hydrocarbon receptor (AHR) in human glioblastoma, an IDO-negative tumor.
Glioma cell lines showed high levels of Kyn and high Trp degradation yet did not express IDO1 or IDO2. Glioma cell lines as well as primary patient tissue expressed TDO, an enzyme normally restricted to the liver. Kyn production was lost in TDO KO glioma cell lines. This the first report of TDO upregulation in cancer. The authors identify other cancers, for instance, ovarian carcinoma and brain metastases of melanoma also with high TDO expression.

As previously described, Kyn suppressed CD4$^+$ and CD8$^+$T cell proliferation and anti-tumor function in PBMC cultures. TDO expressing tumors proliferated faster and provoked reduced anti-tumor immunity in murine models.

Kyn promoted glioma cell survival and a novel autocrine effect of Kyn on tumor migration was observed in Matrigel assays.

Microarray analyses of Kyn treated glioma cells revealed upregulation of several aryl hydrocarbon receptor (AHR) target genes. Kyn induced AHR translocation to the nucleus and reporter activity. Radioligand binding assays confirmed that Kyn is a ligand of AHR. The autocrine effects on tumor growth and the paracrine immunosuppression of TDO and Kyn were dependent on AHR presence.

In clinical studies, high expression of TDO and AHR in glioblastoma sections correlated with less immune infiltration. High TDO, AHR or AHR target gene overexpression correlated with reduced survival in patients.

Taken together with previous studies showing that AHR activation can induce IDO1 and 2, these data reveal a potent feedforward loop of Trp catabolism and AHR activation that promotes tumor progression via autocrine and paracrine mechanisms. An oral TDO inhibitor is being developed and would be an interesting candidate for immunotherapy.
Cancer Exome Analysis Reveals a T Cell-dependent Mechanism of Cancer Immunoediting


Substantial data from pre-clinical and human systems indicates that tumors evoke T cell responses. However, the nature of the target antigens and the role of specific T cell responses in tumor progression and immune escape remain poorly understood. In this important study, Robert Schreiber and colleagues provide new insights into the antigenic basis for tumor immunoediting.

- The authors used high-throughput exome sequencing to identify mutations in chemically-induced sarcomas arising in immunodeficient hosts. While a majority of immunocompetent mice reject transplants of these tumors, escape variants may progress in a small proportion of hosts.

- A small number of the mutations detected in the parental cell line were not found in the escape variants, but were predicted to bind tightly to H2-D^b, the MHC class I allele implicated in tumor rejection.

- The authors isolated a H-2D^b restricted T cell clone from a mouse that had rejected the parental cell line. This clone produced IFN-γ in response to the parental cell line, but not the escape variants. Through integrating the mutational analysis, peptide algorithms, and _in vitro_ T cell specificity, the authors identified a R913L point mutation in spectrin-β2 as the likely target epitope.

- Using the mutant peptide to generate MHC class I tetramers, the authors revealed the generation of T cells specific for mutant spectrin-β2 in mice that rejected the sarcoma cells.
Moreover, enforced expression of the R913L mutation in tumor escape variants promoted T cell mediated rejection.

This data shows that chemically-induced mutations in tumors can be strongly immunogenic and drive either tumor rejection or escape. Interestingly, the C/A and G/T transversions seen in the murine sarcomas are similarly overrepresented in chemical carcinogen associated human tumors, particularly lung cancers that arise in smokers. Furthermore, a study from Tyler Jacks’ laboratory in the same issue of *Nature* provides a second example of immunoediting in the context of genetically engineered cancer.

**IL-10 Elicits IFN-γ-Dependent Tumor Surveillance**


A growing number of immune factors are being identified that can mediate two seemingly opposite functions, depending on the context. Although IL-10 has primarily been viewed as an immunoregulatory cytokine, in this provocative study Martin Oft and colleagues reveal a previously unappreciated role for IL-10 in stimulating tumor destruction through enhanced CD8⁺ cytotoxic T cell responses.

- Unexpectedly, the authors showed that IL-10 deficient mice were more susceptible to chemically-induced skin tumors.
- In wild type tumor bearing mice, the authors noted reduced levels of MHC molecules on antigen presenting cells and only limited numbers of intra-tumoral CD8⁺ cytotoxic T cells.
In a Her2/neu transgenic breast cancer model, treatment with pegylated IL-10 led to rejection of well-established tumors that was CD8^+ T cell dependent. Treatment enhanced MHC expression and augmented intra-tumoral CD8^+ T cell IFN-γ and cytotoxicity, whereas systemic effects were more limited.

In a transplantable tumor model, IL-10 receptor expression on intra-tumoral CD8^+ T cells was higher than CD8^+ T cells present in secondary lymphoid organs or blood. IL-10 enhanced granzyme and IFN-γ expression and the cytotoxicity of splenic CD8^+ T cells stimulated in vitro.

The anti-tumor effects of IL-10 required IFN-γ, which appeared to function primarily in enhancing MHC expression.

A similar association of IL-10 expression with increased levels of MHC, granzyme and IFN-γ were found in human tumor samples.

These data establish a context-dependent role for IL-10 in promoting tumor rejection and support the clinical development of pegylated IL-10 for cancer immunotherapy.
PD-L1 Expression by Metastatic Melanoma Reflects Active Immunity and Improved Survival


Taube et al show that expression of B7-H1 (also known as PDL-1), despite being an immunoregulatory ligand that attenuates T cell responses, may correlate with improved survival in patients with metastatic melanoma. The authors found that B7-H1 expression was directly proportional to the degree of immune infiltration. This observation suggests that, in some cases, immunosuppression within the tumor microenvironment may reflect a response of the tumor to active immunity, rather than a mechanism that initially limits immunity. The study further highlights the complexities of biomarker discovery, revealing important differences in PDL-1 expression across tumor types. Indeed, prior work indicated that PDL-1 is negatively associated with T cell infiltration and patient survival in ovarian carcinoma, esophageal carcinoma, hepatocellular carcinoma, and B cell lymphoma.

- The authors analyzed 150 melanocytic lesions of diverse stages of progression: benign nevi, primary melanomas, and metastatic melanomas. A third of the lesions expressed B7-H1. B7-H1 expression and TIL infiltration did not correlate with any histological subtype or stage of the disease.
• B7-H1 expression on the tumor cells and immune cells correlated with increased immune infiltration, though not all tumors where infiltration was seen expressed B7-H1.

• The most striking finding of the study is the correlation of B7-H1 expression with improved survival in patients with metastatic melanoma, but not in primary melanoma. Given the immunoregulatory function of B7-H1, this finding is counterintuitive. The authors suggest that B7-H1 expression is a response of the tumor to infiltrating immune cells and thereby is a biomarker for the presence of an active immune response.

The authors provide supporting evidence by comparing patient samples where only tumor infiltrating lymphocytes (TIL) are present to those where both B7-H1 and TIL are present. They find that the TIL in B7-H1pos tumors make IFN-\(\gamma\), a known inducer of B7-H1, but the TIL in B7-H1\(^{-}\) tumors do not.

Overall, these data identify a potential prognostic biomarker for metastatic melanoma and emphasize the importance of the PD-L1 pathway in anti-tumor immunity.

**Linking the Abscopal Effect to Anti-Tumor Immunity**


The abscopal effect is a rare phenomenon where tumor regression is seen at a site distant from the site of local therapy with radiation or surgery. Studies in murine tumor models suggest that systemic regression following local radiotherapy may be immune mediated. In this fascinating
case report, Postow and colleagues describe an advanced melanoma patient who achieved a systemic reduction in tumor burden after localized radiotherapy, which was associated with an increase in tumor-specific immune responses. These data provide the first clinical evidence linking the abscopal effect to anti-tumor immunity.

- This metastatic melanoma patient was treated with anti-CTLA-4 mAb (ipilimumab) for disease relapse after surgery and chemotherapy. Ipilimumab treatment led to disease stability, which was correlated with increases in ICOS expression on circulating CD4$^+$ T cells and enhanced T cell and antibody reactivity against the melanoma antigen NY-ESO-1.

- Unfortunately, the patient subsequently suffered disease progression, with an enlarging para-spatial mass and several new lesions. To diminish the pain caused by the para-spatial tumor, the patient was given palliative radiotherapy to the lesion. Strikingly, the patient achieved not only a reduction in the para-spatial mass, but also distant lesions that were not encompassed within the radiation portal underwent regression.

- The systemic anti-tumor effects were linked to further increases in specific antibodies and T cells against NY-ESO-1 and elevation of ICOS expression on CD4$^+$ T cells. Moreover, MHC class II expression on myeloid cells increased, whereas Gr-1 expression decreased.

Overall, these data raise the possibility that immunotherapy with ipilimumab may trigger the abscopal effect and that local radiotherapy may augment cancer immunotherapy. Clinical trials testing the combination of local radiotherapy and immunotherapy are currently underway.
IAP inhibitors enhance co-stimulation to promote tumor immunity

Michael Dougan,1,2,3 Stephanie Dougan,4 Joanna Slisz,5,6 Brant Firestone,5,6 Matthew Vanneman,1,2,3 Dobrin Draganov,1,2,3 Girija Goyal,1,2,3 Weibo Li,7,8 Donna Neuberg,9,10 Richard Blumberg,1 Nir Hacohen,7,8 Dale Porter,5,6 Leigh Zawel,5,6 and Glenn Dranoff1,2,3

1Department of Medical Oncology and 2Cancer Vaccine Center, Dana-Farber Cancer Institute and 3Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115
2Division of Gastroenterology, Hepatology, and Endoscopy, Brigham and Women’s Hospital, Boston, MA 02115
3Oncology Disease Area and 4Developmental and Molecular Pathways Group, Novartis Institutes for Biomedical Research, Cambridge, MA 02139
4Center for Immunology and Inflammatory Diseases, Division of Rheumatology, Allergy, and Immunology, Massachusetts General Hospital, Charlestown, MA 02129
5Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA 02142
6Department of Biostatistics and Computational Biology, Dana–Farber Cancer Institute and 7Department of Biostatistics, Harvard School of Public Health, Boston, MA 02115

The inhibitor of apoptosis proteins (IAPs) have recently been shown to modulate nuclear factor kB (NF-κB) signaling downstream of tumor necrosis factor (TNF) family receptors, positioning them as essential survival factors in several cancer cell lines, as indicated by the cytotoxic activity of several novel small molecule IAP antagonists. In addition to roles in cancer, increasing evidence suggests that IAPs have an important function in immunity; however, the impact of IAP antagonists on antitumor immune responses is unknown. In this study, we examine the consequences of IAP antagonism on T cell function in vitro and in the context of a tumor vaccine in vivo. We find that IAP antagonists can augment human and mouse T cell responses to physiologically relevant stimuli. The activity of IAP antagonists depends on the activation of NF-κB2 signaling, a mechanism paralleling that responsible for the cytotoxic activity in cancer cells. We further show that IAP antagonists can augment both prophylactic and therapeutic antitumor vaccines in vivo. These findings indicate an important role for the IAPs in regulating T cell–dependent responses and suggest that targeting IAPs using small molecule antagonists may be a strategy for developing novel immunomodulating therapies against cancer.

The inhibitor of apoptosis proteins (IAPs) were initially identified as caspase inhibitors capable of blocking both extrinsic and intrinsic apoptotic signals. Recent work has established diverse roles for the IAP family, in which they have been shown to regulate apoptosis through the modulation of NF-κB signaling downstream of several TNF family receptors and to play an essential role in the modulation of FAS-induced cell death (Hu et al., 2006; Leulier et al., 2006; Rigaud et al., 2006; Gaither et al., 2007; Lu et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007, 2008; Vince et al., 2007, 2008; Xu et al., 2007; Bertrand et al., 2008; Mahoney et al., 2008; Matsuzawa et al., 2008; Srinivasula and Ashwell, 2008; Wang et al., 2008; Csomos et al., 2009; Jost et al., 2009). All IAPs contain baculovirus inhibitory repeat domains that mediate protein binding, and several, including cellular IAP-1 (cIAP-1) and cIAP-2, X-linked IAP (XIAP), and melanoma-IAP/Livin, contain RING finger E3 ubiquitin ligase domains, which can cause autoubiquitination as a means of regulating apoptosis (Schile et al., 2008; Srinivasula and Ashwell, 2008). IAPs are regulated endogenously by second mitochondrial-derived activator of caspasess (SMAC), which...
interacts with IAP baculovirus inhibitory repeat domains via a tetrapeptide motif. Several pharmacologic SMAC mimetics have been developed that induce tumor death through binding to the RING domain containing IAPs and leading to ubiquitin-mediated destruction (Gaither et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007; Wang et al., 2008). These pharmacologic SMAC mimetics act as broad antagonists of the RING domain containing IAPs and are actively being investigated as a potential novel class of cancer chemotherapeutics.

In addition to roles in tumor biology, several studies suggest important functions for the IAPs in immunoregulation. XIAP-deficient humans develop X-linked lymphoproliferative disease and were initially reported to lack NKT cells, although the specificity of this finding has recently been challenged (Rigaud et al., 2006; Marsh et al., 2009). XIAP-deficient mice have difficulty controlling Listeria monocytogenes infection and are more susceptible to infection with MHV-68 (mouse herpes virus 68); however, the mechanism for this immunodeficiency is unknown and is not associated with decreased NKT cell function (Bauler et al., 2008; Rumble et al., 2009). cIAP-2 is involved in a recurrent translocation in mucosal-associated lymphoid tissue lymphoma and has been reported to function as an E3 ligase for BCL10 in lymphocytes, although the physiological importance of this activity is unknown (Hu et al., 2006). More recently, the cIAPs were shown to be critical for c-Jun N-terminal kinase activation downstream of CD40 and to negatively regulate alternative NF-κB activation by the BAFF (B cell activation factor of the TNF family) receptor (Matsuzawa et al., 2008; Vallabhapurapu et al., 2008; Zarnegar et al., 2008). These findings position the cIAPs as potentially key regulators of B cell homeostasis, although how the cIAPs regulate B cell–dependent immune responses has, at present, been incompletely explored. In addition to roles in adaptive immunity, the cIAPs and XIAP have been shown to be required for NOD-1 and -2 (nucleotide binding domain) receptor (Matsuzawa et al., 2008; Vallabhapurapu et al., 2008). cIAP-2–deficient mice show altered responses to lipopolysaccharide that may indicate a role for cIAP-2 in inflammatory cytokine-induced apoptosis in macrophages (Conte et al., 2006). Moreover, neuronal apoptosis inhibitor protein (NAIP), a member of both the NOD-like receptor and IAP families, is a component of the inflammasome and is required for control of Legionella pneumophila infections (Diez et al., 2003; Rigaud et al., 2006).

Although evidence now links the IAP family to regulation of both tumor cell survival and immune function, the impact of IAP inhibitors on antitumor immune responses is unknown. In particular, the consequences of IAP antagonism in the key effector cells responsible for antitumor immunity such as CD4+ and CD8+ T cells, NKT cells, and NK cells has not been explored. Given the potential for IAP antagonists to simultaneously induce tumor cell death and modulate immune function, understanding how IAP antagonism might alter nascent antitumor responses and responses to other forms of tumor immunotherapy may have implications for the use of these agents to treat cancer.

In this study, we examine the consequences of IAP antagonism on T cell function both in vitro and in the context of a tumor vaccine in vivo. Unexpectedly, we find that IAPs function as negative co-stimulators during T cell stimulation and that small molecule IAP antagonists can augment both human and mouse T cell responses to physiologically relevant stimuli, including tumor antigens, without producing responses in unstimulated cells.

RESULTS

IAP antagonists have co-stimulatory activity in effector T cells

Effector T cells play a critical role in antitumor immunity. Consequently, the effect of IAP antagonism on T cell function could impact antitumor immune responses occurring in the context of IAP antagonist–mediated tumor cell death. To investigate how IAP antagonists may influence T cell function, we exposed CD4+ T cells isolated from mouse spleens to several IAP antagonists (M1/LB-W-242, M2, and M3) or to a control compound of similar structure (C1/LCV-843; Gaither et al., 2007). We found no evidence of enhanced apoptosis in IAP antagonist–treated T cells regardless of whether the cells were left unstimulated or received a polyclonal–activating signal using antibodies directed against CD3 and CD28 (Fig. 1 A and not depicted). Furthermore, in contrast to tumor cells that are sensitive to IAP antagonism, caspase 3 cleavage was not apparent in IAP antagonist–treated T cells (Fig. 1 B).

Although apoptosis was not affected, IAP antagonists did have a significant effect on T cell function, leading to a substantial increase in T cell numbers after 72 h of culture (P < 0.005 for 5 and 10 μg/ml; Fig. 1 C and Fig. S1). This effect was dependent on strong stimulation and was observed in both anti-CD3– and anti-CD3/CD28–stimulated cultures but not in cultures in which cells were left unstimulated or weakly stimulated (Fig. 1 C and Fig. S1). To determine whether this increase in cell number resulted from an effect on proliferation, we stained CD4+ T cells with the dye CFSE and stimulated them in the presence of IAP antagonists. After 3 d of stimulation, IAP antagonist–treated cultures showed substantially more CFSE dilution than controls, indicating increased proliferation (Fig. 1 D).

In addition to enhanced proliferation, T cells stimulated in the presence of IAP antagonists showed other signs of enhanced activation. IAP antagonist treatment of stimulated CD4+ T cells was associated with changes in surface marker expression characteristic of enhanced activation, including more rapid increases in CD25 and decreases in CD62L and an overall increase in cell size (Fig. 1 E). After 1 d of culture, stimulated T cells treated with M1 reached a level of activation comparable with control cells after 3 d and, in combination with proliferation, produced overall increases in total activated T cells (P < 0.005 for days 1–3; Fig. 1 F).
To exclude the possibility that increased numbers of CD4+CD25+ cells represented an increase in the T reg cell population, which has an overlapping surface phenotype with activated T cells (Fontenot et al., 2005), we stimulated CD4+ T cells isolated from mice expressing the transcription factor FOXP3 linked to GFP; FOXP3 is required for T reg cell development and maintenance, and in these mice, all T reg cells are marked with GFP (Fontenot et al., 2005). As was observed in wild-type animals, CD4+ T cells isolated from FOXP3-GFP mice showed increased CD25 expression upon stimulation in the presence of IAP antagonists; however, after 2 d in culture, the number of FOXP3-GFP–expressing cells remained essentially unchanged, demonstrating that IAP antagonism does not selectively expand this population (Fig. 1, G and H).

We next tested whether IAP antagonists could enhance cytokine production from stimulated CD4+ T cells in a manner similar to their effect on other markers of T cell activation. Consistent with enhanced activation, isolated CD4+ T cells treated with IAP antagonists and stimulated by

Figure 1. IAP antagonists enhance mouse T cell proliferation and activation. (A–H) CD4+ T cells were positively selected from mouse spleens using magnetic beads and stimulated with 10 µg/ml plate-bound anti-CD3 (or as indicated) and 2 µg/ml anti-CD28 in the presence of IAP antagonist (M1) or control compound (C1) at 500 nM. (A) 5 × 10⁵ CD4+ T cells were stimulated for 24 h. Annexin V and 7AAD staining were determined by flow cytometry. (B) Immunoblots for ZAP-70 and caspase 3 on total cell lysates from CD4+ T cells stimulated as indicated. (C and D) 10⁵ CD4+ T cells were stimulated as indicated. (C) After 72 h, relative cell numbers were determined using CellTiter-Glo luminescent cell viability assay (Promega) and normalized to unstimulated cultures treated with C1. (D) Cells were labeled with CFSE before stimulation, and fluorescence was measured after 72 h by flow cytometry. (E) 5 × 10⁵ CD4+ T cells were stimulated for the indicated periods of time, and CD25, CD62L, and forward scatter (FSC) were determined by flow cytometry. (F) Quantification of E using cell numbers determined using CellTiter-Glo luminescent cell viability assay (Promega) and normalized to unstimulated cultures treated with C1. (G) Cells were labeled with CFSE before stimulation, and fluorescence was measured after 72 h by flow cytometry. (H) Quantification of G using three replicates per group. (A–H) Error bars represent SEM. Results are representative of at least two independent experiments.
Increasing concentrations of anti-CD3 in the presence or absence of 2 μg/ml anti-CD28 produced substantially more IL-2 than similarly cultured cells treated with control compound ($P < 0.005$ for 5 and 10 μg/ml; Fig. 2 A). The effect of IAP antagonism was dose dependent and was observed with three distinct compounds ($P = 0.0002$ for M1; Fig. 2 B and Fig. S2). In addition to effects on IL-2 production, levels of both IL-4 and IFN-γ were increased in IAP antagonist–treated cultures (unpublished data). Isolated CD8+ T cells stimulated with anti-CD3/CD28 also responded to IAP antagonist treatment with an increase in cytokine production, indicating a general activation-enhancing effect of IAP antagonists on T cells (Fig. 2 C).

T cells are activated physiologically by the recognition of antigenic peptides bound to MHC on the surface of antigen-presenting cells (Heemels and Ploegh, 1995). Consequently, we next sought to examine IAP antagonism in the context of peptide-restricted T cell activation. OTI CD8+ T cells, which recognize the OVA peptide SIINFEKL bound to MHC class I (Hogquist et al., 1994), were stimulated with peptide-loaded DCs in the presence of either the IAP antagonist M1 or control compound. As was observed in the context of activating antibodies, IAP antagonist treatment led to a dose-dependent enhancement in cytokine production with effects ranging from five- to sevenfold ($P < 0.0001$ for all M1 concentrations; Fig. 2 D); furthermore, IAP antagonists increased cytokine production at a range of peptide concentrations and when DCs were pulsed with whole OVA protein (Fig. S3; Hogquist et al., 1994).

In addition to peptide-reactive CD4+ and CD8+ T cells, both NKT cells and NK cells have been shown to play important roles in certain antitumor immune responses (Dougan and Dranoff, 2009). Consequently, we wondered how these cell types would respond to treatment with IAP antagonists. Consistent with the effect of IAP antagonists on peptide-specific responses, spleen cells stimulated with the NKT cell–specific agonist α-galactosylceramide produced significantly more IFN-γ than cells treated with control compound ($P = 0.007$; Fig. 2 E). This effect was specific to stimulated cultures, as no cytokine was produced by cultures treated with IAP antagonists alone (Fig. 2 E). NK cell responses were also sensitive to IAP antagonism, as IAP antagonist–treated NK cells produced substantially more granulocyte-macrophage CSF (GM-CSF) after exposure to NKG2D ligand expressing YAC-1 cells, although IAP antagonist treatment alone had no effect on GM-CSF production ($P < 0.0001$; Fig. 2 F; Raulet et al., 2001).

Collectively, these findings indicate that IAP antagonists can augment lymphocyte co-stimulation. As is the case with co-stimulatory signals delivered through surface receptors (Greenwald et al., 2005), in the absence of antigen stimulation, the IAP antagonists have no effect on any of the measured parameters of T cell activation, including proliferation, surface marker expression, and cytokine production; however, when T cells are given a strong activating signal, either with antibodies or peptide in the context of MHC, IAP antagonism leads to a significant enhancement in T cell function. Similar findings were observed with both NKT cells and NK cells, suggesting that IAP antagonists can broadly co-stimulate multiple cell types involved in antitumor responses and may be useful in augmenting antitumor immunity.

**Human T cells are sensitive to IAP antagonists**

We next decided to test whether our observations in mouse T cells could be generalized to human T cells. We first isolated human CD4+ T cells from the peripheral blood of healthy donors and stimulated them with anti-CD3/anti-CD28 in a manner analogous to our mouse T cell stimulations. Under these conditions, IAP antagonist treatment was associated with both dose-dependent enhancements in cytokine production ($P < 0.0001$; Fig. 3, A and B) and changes in surface marker expression and cell size (Fig. 3 C) similar to those observed with mouse cells. After 3 d of stimulation, activated CD25+CD62L− T cells represented nearly 29% of IAP antagonist–treated cultures compared to 13% in control cultures ($P = 0.009$).

**Figure 2.** IAP antagonists enhance the stimulation of multiple immune effectors. (A–C) CD4+ or CD8+ T cells were isolated as in Fig. 1. (A) 105 CD4+ T cells were isolated and stimulated with anti-CD3 as indicated and 2 μg/ml anti-CD28 for 72 h. M1 and C1 were used at 500 nM. (B) 105 CD4+ T cells were isolated and stimulated with 10 μg/ml anti-CD3 and 2 μg/ml anti-CD28 for 48 h in the presence of M1 or control compound at the indicated concentrations. (C) 105 CD8+ T cells were isolated and stimulated as in B for 48 h. M1 and C1 were used at 500 nM. (D) Human CD4+ T cells were isolated and stimulated with 1 μg/ml anti-CD3. (E) 2 x 105 DX5+ NK cells were positively selected from mouse spleens using magnetic beads and stimulated for 48 h by co-culture with 4 x 105 YAC-1 cells in the presence of M1 or control compound at 500 nM. (A–F) Cytokines were measured by ELISA. Error bars represent SEM. Results are representative of at least two independent experiments.
antagonist–treated cells compared with 18% of control cells; however, in contrast to mouse cells, the total fraction of CD25+ T cells was only modestly increased (73.5 vs. 59.8%; Fig. 3 C). Also consistent with findings in the mouse, human cells were not activated by IAP antagonist treatment alone, further demonstrating a role for IAP antagonists in T cell co-stimulation (Fig. 3 A).

We also assessed the ability to IAP antagonists to augment activation of T cells stimulated by the superantigen staphylococcus enterotoxin B (SEB), in addition to stimulation through activating antibodies. When human PBMCs were incubated with SEB, IAP antagonists enhanced cytokine production by as much as fivefold (P < 0.0001 for M1 and M2; Fig. 3 D). Collectively, these results indicate that IAP antagonist treatment can co-stimulate T cell activation in both mice and humans.

Alternative NF-κB signaling is required for IAP antagonist–induced T cell co-stimulation

The IAPs have been implicated in a wide range of signaling pathways that could modulate T cell activation (Srinivasula and Ashwell, 2008). Although caspases have a well-described role in T cell activation (Bidère et al., 2006) and several of the IAPs are known to regulate caspases, we found that relieving caspase inhibition does not appear to be the primary mechanism underlying IAP antagonist activity in T cells. In addition to failing to activate caspase 3, IAP antagonists had an equivalent effect on T cell activation regardless of whether the cells were pretreated with the caspase inhibitor ZVAD-fmk (Figs. 1 B and 4 A).

Because cIAP-2 has been reported to regulate NF-κB activation through the ubiquitination and degradation of BCL10 (Hu et al., 2006), we also assessed the effects of the IAP antagonists on BCL10 abundance. Although a decrease in BCL10 was observed upon T cell stimulation, this change was not altered by treatment with IAP antagonists, suggesting that the regulation of BCL10 abundance is not the principal mechanism of IAP antagonist activity in T cells (Fig. 4 B).

In tumor cells, IAP antagonist–mediated apoptosis depends on the modulation of both alternative and classical NF-κB signaling downstream of TNF family receptors. In these systems, the cIAPs constitutively down-regulate NF-κB–inducing kinase (NIK), blocking alternative NF-κB activation; however, the cIAPs are also indispensable in classical NF-κB activation, promoting the association of receptor-interacting protein with TAK1 (transforming growth factor β–activated kinase 1) through receptor-interacting protein ubiquitination (Gaither et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007, 2008; Vince et al., 2007, 2008; Mahoney et al., 2008; Wang et al., 2008). Regulation of TNF signaling by IAPs is conserved in Drosophila melanogaster, where DIAP2 (Drosophila IAP2) plays a critical role in the inducible pathway, an immune response pathway orthologous to TNF signaling in mammals (Leulier et al., 2006).

Based on the evidence linking IAPs to NF-κB modulation, as well as the importance of NF-κB in T cell biology, we examined classical and alternative NF-κB signaling in T cells during IAP antagonist treatment (Karin and Lin, 2002). Stimulated mouse CD4+ T cells treated with M1 showed enhanced p100 processing to p52, as well as enhanced p52 and RelB nuclear localization (Fig. 4, C and D); in addition, M1 had moderate effects on IkBα (inhibitor of NF-κB α) levels (Fig. 4 C). Activation of alternative NF-κB was also apparent in unstimulated T cells exposed to IAP antagonists, demonstrating that, similar to their effects in tumor cells, IAP antagonists are sufficient to activate alternative NF-κB in T cells even in the absence of antigen signaling (Fig. 5 B and Fig. S4).

The observation of alternative NF-κB signaling in IAP antagonist–treated T cells suggests a role for this pathway in IAP antagonist–mediated co-stimulation. To directly test the importance of alternative NF-κB signaling in IAP antagonist–treated T cells, we evaluated responses to M1 in NIK-deficient lymphoplasia
In principle, several IAPs could function as targets for IAP antagonists even in the absence of signaling through their receptors (Varfolomeev et al., 2007, 2008), degradation of the cIAPs was associated with increased IFN-γ production, suggesting a role of the cIAPs in negatively regulating activation (cIAP-1 KD1, P = 0.001; cIAP-2 KD2, P = 0.009; cIAP-2 KD3, P < 0.0001; Fig. 5 D).

Through the T cell receptor was not required for cIAP reduction, as loss of the cIAPs occurred both in the presence and absence of stimulation (Fig. 5 A and Fig. S6). Consistent with our previous findings (Fig. 3 and Fig. 4, C–F) and other published work (Varfolomeev et al., 2007, 2008), degradation of the cIAPs in human T cells was associated with activation of alternative NF-κB signaling (Fig. 5 B); as was observed with mouse T cells, alternative NF-κB activation by IAP antagonists occurred in both stimulated and unstimulated cells (Fig. 5 B and Fig. S6).

To specifically address the role of the cIAPs in human T cells, we infected primary human CD4+ T cells with lentiviral constructs encoding short hairpin RNA directed against cIAP-1 and cIAP-2 (KD1–KD3; Moffat et al., 2006). Several of the evaluated constructs led to efficient knockdown of their targets in primary T cells (Fig. 5 C). In the mouse, loss of one cIAP leads to a compensatory increase in the other cIAP (Conze et al., 2005); however, transient knockdown of cIAP-1 and cIAP-2 in human T cells led to only a minor compensatory up-regulation of the other cIAP (Fig. 5 C). Efficient knockdown of either cIAP-1 or cIAP-2 in stimulated T cells was associated with increased IFN-γ production, suggesting a role of the cIAPs in negatively regulating activation (cIAP-1 KD1, P = 0.001; cIAP-2 KD2, P = 0.009; cIAP-2 KD3, P < 0.0001; Fig. 5 D).

**cIAP-2 is regulated during T cell co-stimulation**

These findings indicate a role for the cIAPs as regulators of T cell activation through their ability to negatively regulate alternative NF-κB signaling. Given recent evidence that TNF family ligands can induce cIAP-1 degradation downstream of their receptors (Varfolomeev et al., 2008), we hypothesized that the cIAPs may be regulated as part of...
T cell co-stimulation. Activated T cells express several TNF family co-stimulatory receptors that activate alternative NF-κB; among these, glucocorticoid-induced TNF receptor (GITR) is under active study in the context of tumor immunity, and thus we selected this molecule for more detailed characterization (Watts, 2005). Activation of human T cells with anti-CD3/CD28 led to cIAP-2 up-regulation; however, additional co-stimulation using anti-GITR antibodies was associated with a decrease in cIAP-2 concurrent with increased IFN-γ production and loss of p100 (Fig. 5 E and not depicted). Levels of cIAP-1 were not clearly changed in anti-GITR–stimulated cells during the time points examined (unpublished data). These findings suggest a physiological role for cIAP-2 downstream of GITR co-stimulation.

In vivo exposure to IAP antagonists enhances T cell susceptibility to stimulation but does not lead to generalized T cell activation

We next sought to evaluate the consequences of systemic delivery of the IAP antagonist M1 on T cell and NK cell populations in the spleen. Consistent with our findings in cell culture, in the absence of additional signaling, neither CD4+ nor CD8+ T cell numbers were altered by M1 administration, nor were any clear effects observed on NK cells or NK1.1+ T cells (Fig. 6, A and B). Although, as anticipated, broad T cell activation did not occur when mice were treated with M1, a small increase in the baseline number of CD69+ T cells was observed, possibly indicating a systemic increase in T cell activation (P = 0.03; Fig. 6 C). In contrast to the small increase in activation markers observed in unstimulated cells, when CD4+ T cells were isolated from M1-treated mice and stimulated ex vivo with anti-CD3/CD28 antibodies (in the absence of added inhibitors), these cells showed a hyperresponsive phenotype, producing substantially more IL-2 than cells isolated from control animals (P = 0.005; Fig. 6 D). These findings provide further evidence for a co-stimulatory effect of IAP antagonism and indicate that IAP antagonists may be able to augment immune responses in vivo.

IAP antagonists can enhance the potency of tumor vaccines

Based on these findings, we hypothesized that IAP antagonists could function to augment tumor vaccine–induced responses. To evaluate IAP antagonist activity in vivo, we first used a suboptimal tumor cell vaccine in which irradiated B16 mouse melanoma cells are used to protect against live B16 challenge in syngeneic C57BL/6 mice. This approach has low intrinsic potency, but vaccine efficacy can be enhanced through a variety of immune manipulations, making it a useful system for studying strategies for augmenting antitumor immunity (Dranoff et al., 1993; Jinushi et al., 2007). This system is further...
IAP inhibitors enhance co-stimulation | Dougan et al.

Combined vaccine is mediated by an increase in T cell immunity (P = 0.003; Fig. 7 D; Jinushi et al., 2007). These findings establish as proof of principle that IAP antagonists can function as immunostimulants.

We next sought to assess the ability of IAP antagonists to augment immune responses in the context of a more efficacious tumor vaccine. Irradiated B16 vaccines comprised of cells engineered to secrete GM-CSF (GVAX) can provide potent antitumor immunity, completely protecting mice from subsequent tumor challenge (Dranoff et al., 1993, Jinushi et al., 2007). However, when used on established tumors in a therapeutic setting, GVAX slows tumor growth but does not result in tumor eradication, making this an appropriate model to study IAP antagonists in the setting of a potent but incompletely effective vaccine (Jinushi et al., 2007).

We first examined B16 responses in mice vaccinated with either GVAX or GVAX in combination with M1. In combination-treated mice, IAP antagonist treatment was begun on day 1 and continued until day 6. On day 7, the lymphocytes are appropriate because we found that the IAP antagonists do not appear to have direct effects on B16 cells in culture. IAP antagonist treatment does not alter B16 proliferation in vitro (Fig. 7 A); furthermore, IAP antagonists do not enhance apoptosis or caspase cleavage in B16 cells after irradiation (Fig. 7 B and Fig. S7).

Consistent with the effect of IAP antagonists in vitro and previous experiments studying B16 vaccines (Dranoff et al., 1993), mice treated with either the IAP antagonist M1 or irradiated B16 melanoma cells alone showed no delay in tumor growth compared with controls after B16 challenge. However, combination treatment of mice with M1 and irradiated B16 cells led to a 65% reduction in B16 growth after challenge (P = 0.02; Fig. 7 C). This reduction was associated with a significant increase in the frequency of CD8+ T cells specific for the B16 antigen TRP-2 (Jinushi et al., 2007) in the spleens of mice treated with the combination therapy compared with animals treated with irradiated B16 alone, suggesting that, in part, the efficacy of the combined vaccine is mediated by an increase in T cell immunity (P = 0.003; Fig. 7 D; Jinushi et al., 2007). These findings establish as proof of principle that IAP antagonists can function as immunostimulants.

We next sought to assess the ability of IAP antagonists to augment immune responses in the context of a more efficacious tumor vaccine. Irradiated B16 vaccines comprised of cells engineered to secrete GM-CSF (GVAX) can provide potent antitumor immunity, completely protecting mice from subsequent tumor challenge (Dranoff et al., 1993, Jinushi et al., 2007). However, when used on established tumors in a therapeutic setting, GVAX slows tumor growth but does not result in tumor eradication, making this an appropriate model to study IAP antagonists in the setting of a potent but incompletely effective vaccine (Jinushi et al., 2007).

We first examined B16 responses in mice vaccinated with either GVAX or GVAX in combination with M1. In combination-treated mice, IAP antagonist treatment was begun on day 1 and continued until day 6. On day 7, the lymphocytes are appropriate because we found that the IAP antagonists do not appear to have direct effects on B16 cells in culture. IAP antagonist treatment does not alter B16 proliferation in vitro (Fig. 7 A); furthermore, IAP antagonists do not enhance apoptosis or caspase cleavage in B16 cells after irradiation (Fig. 7 B and Fig. S7).

Consistent with the effect of IAP antagonists in vitro and previous experiments studying B16 vaccines (Dranoff et al., 1993), mice treated with either the IAP antagonist M1 or irradiated B16 melanoma cells alone showed no delay in tumor growth compared with controls after B16 challenge. However, combination treatment of mice with M1 and irradiated B16 cells led to a 65% reduction in B16 growth after challenge (P = 0.02; Fig. 7 C). This reduction was associated with a significant increase in the frequency of CD8+ T cells specific for the B16 antigen TRP-2 (Jinushi et al., 2007) in the spleens of mice treated with the combination therapy compared with animals treated with irradiated B16 alone, suggesting that, in part, the efficacy of the combined vaccine is mediated by an increase in T cell immunity (P = 0.003; Fig. 7 D; Jinushi et al., 2007). These findings establish as proof of principle that IAP antagonists can function as immunostimulants.
nodes that drain the vaccine site were harvested and cultured with irradiated B16 cells. Lymph node cell cultures from mice treated with combination therapy produced substantially more IFN-γ in response to B16 cells than did cells taken from mice treated with either M1 or GVAX alone (P = 0.03; Fig. 8 A). Furthermore, significantly fewer B16 cells remained in the lymph node cultures derived from combination-treated mice compared with cultures from GVAX- or M1-treated animals (P = 0.03; Fig. 8 B). This loss of B16 cells was likely caused by enhanced cytotoxicity by cells from combination-treated animals because these cultures showed enhanced B16 cell killing in chromium release assays (unpublished data). In addition to lymph node responses, IFN-γ production from NK cells harvested from the spleens of vaccinated mice and co-cultured with YAC-1 cells was also enhanced in combination-treated animals compared with mice treated with GVAX alone (P < 0.0001; Fig. 8 C). Lymph nodes harvested from combination-treated animals were only marginally larger than lymph nodes from GVAX-treated mice, though, and contained similar numbers of B cells, T cells, and DCs, as well as activated and regulatory T cell subsets (Fig. 8A). IFN-γ production was higher in lymph node cultures from combination-treated mice even when the differences in cell number were taken into account (unpublished data).

We next examined whether IAP antagonist treatment could augment the effect of GVAX on tumor growth. Mice were vaccinated 1 d after B16 challenge with either GVAX alone or GVAX followed by daily treatment with M1 for 1 wk. Combination-treated mice showed significantly decreased tumor growth rates compared with animals treated with GVAX or M1 alone (P < 0.05 from day 10–25), although median survival was not prolonged (Fig. 8, D and E). Although additional studies to identify optimal dosing regimens are required, these results confirm the immunomodulatory activity of the IAP antagonists and establish the ability of these drugs to improve responses to tumor vaccines in both prophylactic and therapeutic settings.

**DISCUSSION**

Collectively, our findings establish the IAPs as important regulators of T cell activation and define IAP antagonists as a novel class of immunomodulating agent. Unlike conventional adjuvants, IAP antagonists act in a co-stimulatory capacity, enhancing immune responses to physiological immune signals in both mice and humans, while lacking intrinsic stimulatory capacity. The co-stimulatory effect is striking in T cells where IAP antagonism in the context of stimulation leads to enhanced cytokine secretion, as well as increased proliferation and expression of activation markers.

IAP antagonism appears to co-stimulate T cells by blocking the ability of the cIAPs to inhibit alternative NF-κB signaling, likely through their ability to regulate NIK, as has been shown in a variety of other cell types (Gaither et al., 2007; Lu et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007, 2008; Vince et al., 2007, 2008; Bertrand et al., 2008; Mahoney et al., 2008; Vallabhappurapu et al., 2008; Zarneger et al., 2008). T cells from mice harboring spontaneous mutations in NIK are resistant to IAP antagonism, and decreases in cIAP-1 and cIAP-2 after antagonist treatment are associated with rapid activation of alternative NF-κB. Similarly, knockdown of cIAP-1 and cIAP-2 enhances cytokine production from stimulated T cells.

In human T cells, cIAP-2 is regulated after signaling downstream of GITR. This finding indicates a potential physiological role for the IAPs in the regulation of GITR co-stimulation, which is known to depend in part on alternative NF-κB (Watts, 2005). Given the range of TNF family receptors now known to signal through the IAPs, this finding further suggests that in T cells, the IAPs may play a role downstream of TNF receptor co-stimulation more broadly. If this hypothesis is born out, then IAP antagonists could function as activators of multiple co-stimulatory receptors simultaneously, while bypassing any requirement for co-stimulatory receptor ligation. Such co-stimulatory activity by a small molecule may be useful in a wide variety of immunotherapies, including vaccine development and the treatment of immunodeficiencies. Indeed, several different agonistic.
mAbs against TNF family receptors are currently under investigation as targets for immunotherapy for cancer; these include antibodies to GITR, CD134 (OX40), and CD137 (4-1BB), with anti-CD137 antibodies now in phase I/II testing (Dougan and Dranoff, 2009).

In mice, we have shown that in vivo administration of IAP antagonists can augment the efficacy of both prophylactic and therapeutic tumor vaccines. IAP antagonist treatment is associated with systemic hyperresponsive T cells in the absence of overt autoimmunity or expansion of the T cell compartment. After vaccination, IAP antagonist delivery leads to augmented antitumor responses that are associated with delayed tumor growth and prolonged survival. These findings demonstrate that the effects of IAP antagonism observed in culture can, at least in part, be harnessed in vivo to modulate immune responses. Future work will be necessary to fully optimize this approach, in particular focusing on alternative conditions for IAP antagonist delivery and the introduction of optimal antigenic signals. Our results in cell culture suggest that certain threshold levels of stimulation substantially increase the efficacy of IAP antagonists, as indicated by the change in the magnitude of the IAP antagonist effect during anti-CD3 dose titration (Figs. 1C and 2A). Moreover, although we have found evidence for apoptosis regulation by the IAPs in cell types examined in these experiments, further investigations may well reveal circumstances under which the antia apoptotic activity of the IAPs is indispensable for T cell survival or function.

The unanticipated activity of the IAP antagonists in T cells and other effectors of antitumor immunity also enables a novel approach to chemotherapy for cancer. IAP antagonists could have a synergistic effect on tumors. Through direct cytotoxicity, IAP antagonists could increase tumor cell death, leading to increased presentation of tumor antigens to the immune system. At the same time, we have now demonstrated that inhibiting IAPs removes a physiological signaling brake, allowing for enhanced responses from both CD4+ and CD8+ T cells as well as other key antitumor effector cells, including NKT cells and NK cells.

MATERIALS AND METHODS

Animals. C57BL/6 and BALB/c wild-type mice were purchased from Taconic or The Jackson Laboratory or bred in house. Aly/aly mice were obtained from G. Benichou (Massachusetts General Hospital, Boston, MA; Miyawaki et al., 1994). FOXP3-GFP knockin mice were obtained from A. Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY; Fontenot et al., 2005). All animal experimentation was performed in accordance with institutional guidelines and the review board of Harvard Medical School, which granted permission for this study, and was approved by the Association for Assessment and Accreditation of Laboratory Animal Care-accredited Dana-Farber Cancer Institute Institutional Animal Care and Use Committee.

Antibodies. All antibodies used for flow cytometry were obtained from BD. BCL10 (rabbit pAb; Cell Signaling Technology), caspase 3 (rabbit pAb; Cell Signaling Technology), human CD3 (mouse mAb clone HIT3a; BD), mouse CD3 (hamster mAb clone 145-2C11; BD), human CD28 (mouse mAb clone CD28.2; BD), mouse CD28 (hamster mAb clone 37.15; BD), cIAP-1 (goat pAb; R&D Systems), cIAP-2 (mouse mAb clone 315304; R&D Systems), GAPDH (mouse mAb clone MAB374; Millipore), GITR, (mouse mAb DTSD3; Milenyi Biote), IκB-α (mouse mAb clone 112B2; Cell Signaling Technology), p100/p52 (rabbit pAb; Cell Signaling Technology), RelB (rabbit pAb; Cell Signaling Technology), USF-2 (rabbit pAb; Santa Cruz Biotechnology, Inc.), XIAP (clone 28; BD), and ZAP-70 (rabbit mAb clone D1C10E; Cell Signaling Technology) were also used.

Flow cytometry and immunoblotting. Single cell suspensions were made from resected spleens or lymph nodes by mechanical disruption; red blood cells were removed by hypotonic lysis. CD4, CD8, or DX5+ cells were purified either from organs suspensions (mouse) or peripheral blood (human) using magnetic beads conjugated to the indicated antibodies (Milenyi Biote); purifications were performed according to manufacturer’s instructions. Cells were then washed and stained for 30 min on ice with the indicated antibodies in PBS with 1% inactivated fetal calf serum; for most experiments, Fc receptors were blocked using an unconjugated Fc-blocking antibody (BD) 15 min before staining. For Western blot analysis, cells were lysed in 1% NP-40 or using a cytoplasmic/nuclear fractionation kit (Thermo Fisher Scientific). Protein concentration was quantified by bicinchoninic acid assay (Thermo Fisher Scientific), and 30 µg of protein was loaded per lane onto 12% acrylamide gels. SDS-PAGE was followed by transfer to nitrocellulose and immunoblotting using the indicated primary antibodies. Secondary antibodies conjugated to alkaline phosphatase were purchased from Jackson ImmunoResearch Laboratories, Inc. and used at 1:5,000 dilution.

ELISA. All ELISA kits were purchased from BD and used according to the manufacturer’s instructions.

Lentiviral knockdown. 2 × 10^6 CD4+ T cells isolated by positive selection from human peripheral blood were infected by spin transduction with highly concentrated lentiviral particles (Broad Institute and Sigma-Aldrich) using a multiplicity of infection of 3. 2 µg/ml puromycin was added 48 h after infection. Cells were analyzed 4–6 d later. Infections and subsequent cultures were performed in the presence of 2 µg/ml plate-bound anti-CD3, 2 µg/ml anti-CD28, and 20 U/ml recombinant human IL-2 in 100 µl RPMI. cIAP-1 KD1, 5′-GCCGATAATTTCGCTTGGTGTGCTCAGGAAAGCCAAAAGACAATTCGCGC-3′; cIAP-1 KD2, 5′-GCTGCGGCGAACCATCTCACAACCTCGAGTTGTAAGTGGCCGACG-3′; cIAP-1 KD3, 5′-TGTTGAATAATCTGCTCTGGAAACCTGACTTCCACGAACTTTAACCA-3′; cIAP-2 KD1, 5′-CACTGTTGCTAATCTTCTCGAGGATGAAAGAAATGTACGAACTG-3′; cIAP-2 KD2, 5′-GCAGACTCATCAATTAATTCATCCATCGAGGATGAAAGAAATGTACGAACTG-3′; and cIAP-2 KD3, 5′-GCACCTACAAAACACAATATTCCATCGAGGATGAAAGAAATGTACGAACTG-3′ were used.

Anti-GITR co-stimulation. 3 × 10^6 CD4+ human T cells isolated from the peripheral blood by positive selection were incubated with either 2 or 10 µg/ml of plate-bound anti-CD3 and 2 µg/ml anti-CD28 and 24 U/ml recombinant human IL-2 in 100 µl RPMI. After 24 h, T cells were either continued on anti-CD3/CD28 stimulation for an additional 14–24 h or stimulated with plate-bound anti-CD3/CD28 and 5 µg/ml anti-GITR for the same period of time. Co-stimulation was verified by measuring cytokine production in the culture supernatants by ELISA.

Ex vivo stimulation. Mice were treated daily with M1 by gastric lavage at a dose of 750 µg per day. After 1 wk, mice were euthanized, and CD4+ T cells were isolated by positive selection using magnetic beads. 10^6 CD4+ T cells were incubated with 5 µg/ml of plate-bound anti-CD3 and 2 µg/ml anti-CD28 for 48 h in 500 µl RPMI. After 48 h, T cells were either continued on anti-CD3/CD28 stimulation for an additional 4–24 h or stimulated with plate-bound anti-CD3/CD28 and 5 µg/ml anti-GITR for the same period of time. Co-stimulation was verified by measuring cytokine production in the culture supernatants by ELISA.

Prophylactic B16 vaccine. On days 0 and 7, mice were given a subcutaneous injection containing 5 × 10^6 irradiated (3.5 krad) B16 cells in conjunction with oral administration of M1 or control compound at either 150 or 30 mg/kg. On day 14, mice were challenged with a subcutaneous injection containing 5 × 10^5 live B16 cells.

B16 GVAX. On day 0, mice were challenged with a subcutaneous injection containing 2 × 10^5 live B16 cells. On day 1, mice received a subcutaneous
injection containing $5 \times 10^7$ irradiated (3.5 krad) B16 cells engineered to secrete GM-CSF (GVAX). On days 2–6, mice were given M1 at 1,000 µg per day by gastric lavage.

**Enzyme-linked immunosorbent spot.** 2.5 × 10^8 CD8⁺ T cells isolated by positive selection from vaccinated mouse spleens were incubated with 10^6 irradiated syngeneic mouse splenocytes pulsed with TRP-2 peptide (180–188: SYVDFPVWL) or vehicle control (DMSO) for 24 h in 100 µl RPMI media.

**Ex vivo cultures.** Cells were isolated from the vaccine site draining lymph node or from the spleens of vaccinated mice by mechanical disruption; red blood cells were removed by hypotonic lysis. For B16 cultures, cells were incubated and analyzed as described in the figure legend. NK cells were isolated by DX5⁺ cell purification using magnetic beads (Miltenyi Biotec) and cultured as described in the figure legend.

**Statistics.** Two sample comparisons used the t test with pooled variance if there was no evidence of inhomogeneity of variances between groups. If the variances were unequal, the exact Wilcoxon rank sum test was performed with matched pairs and Wilcoxon signed-rank test was used. Every effort was made to keep testing consistent across related experiments. For comparisons of more than two groups, analysis of variance was used if there was no evidence of inhomogeneity of variance; the Kruskal-Wallis test was the nonparametric alternative. Tumor growth experiments were analyzed using mixed model analysis of variance; the nonparametric alternative to the Kruskal-Wallis test, was used. Every effort was made to keep testing consistent across related experiments. For comparisons of more than two groups, analysis of variance was used if there was no evidence of inhomogeneity of variance; the Kruskal-Wallis test was the nonparametric alternative. Tumor growth experiments were analyzed using mixed model analysis of variance and the Welch’s t test.

**Online supplemental material.** Fig. S1 shows relative cell counts and IL-2 production from IAP antagonist–treated CD4⁺ T cells stimulated with anti-CD3 at several concentrations as indicated. Fig. S2 shows IL-2 production from CD4⁺ T cells stimulated with anti-CD3/CD28 and treated with one of three distinct IAP antagonists, a control compound of similar structure, or vehicle. Fig. S3 shows cytokine production from IAP antagonist–treated OTI T cells stimulated with the OTI-specific agonist peptide SIINFEKL or whole OVA protein presented by formalin-fixed DCs at the indicated concentrations. Fig. S4 shows nuclear RelB and p52 levels in unstimulated, pu- rified CD4⁺ T cells exposed to IAP antagonist or control compound. Fig. S5 shows the strategy used to purify naive T cells from +/aly and aly/aly spleens and confirms the purity of the post-sort populations. Fig. S6 shows cIAP-1 and cIAP-2 levels in unstimulated, purified, human CD4⁺ T cells exposed to IAP antagonist or control compound. Fig. S7 shows annexin V/7AAD (7-aminomercycyanide) staining and caspase 3 cleavage in cultured B16 cells exposed to IAP antagonists. Fig. S8 shows immune populations in the draining lymph node of mice vaccinated with GVAX or GVAX plus daily dosing of an IAP antagonist. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101123/DC1.

We thank Giles Benichou for providing aly/aly mice.

This work was supported by the Dana-Farber Cancer Institute/Novartis Program in Drug Discovery (grant to G. Dranoff) and grant F30AG030298 from the National Institute on Aging (to M. Dougan). G. Dranoff is a grantee of and consultant to the Novartis Institute of Biomedical Research. J. Sizs, B. Firestone, D. Porter, and L. Zawel are employees of the Novartis Institute of Biomedical Research. The authors have no additional conflicts of interest.

**Submitted:** 4 June 2010  
**Accepted:** 10 August 2010

**REFERENCES**


doi:10.1073/pnas.0907131106
inhibitor of apoptosis protein DIAP2 functions in innate immunity and
is essential to resist gram-negative bacterial infection. Mol. Cell. Biol.
26:7821–7831. doi:10.1128/MCB.00548-06
Han, and H. Wu. 2007. XIAP induces NF-kappaB activation via the
BIR1/TAB1 interaction and BIR1 dimerization. Mol. Cell. 26:689–
702. doi:10.1016/j.molcel.2007.05.008
Mahoney, D.J., H.H. Cheung, R.L. Mrad, S. Plenchette, C. Simard, E.
Enverre, V. Arora, T.W. Mak, E.C. Lacasse, J. Waring, and R.G.
Korneluk. 2008. Both cIAP1 and cIAP2 regulate TNFalpha-mediated
doi:10.1073/pnas.0711121105
Marsh, R.A., J. Villanueva, M.O. Kim, K. Zhang, D. Marmer, K.A. Risma,
inherited lymphopenoproliferative disease due to BIRC4 mutation have normal
doi:10.1016/j.clim.2009.03.017
Wang, D.A. Vignali, E. Gallagher, and M. Karin. 2008. Essential cyto-
plasmic translocation of a cytokine receptor-assembled signaling com-
Miyawaki, S., Y. Nakamura, H. Suzuki, M. Koba, R. Yasunari, S. Ikehara,
lack of lymph nodes accompanied by immunodeficiency in mice. Eur.
Moffat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.
Mofat, J., D.A. Grueneberg, X. Yang, S.Y. Kim, A.M. Kloepfer, G.
Hinkle, B. Pequignot, T. Eisenhaure, L. Li, M. Peyton, J. Minna, P.