IL-12-based vaccination in melanoma: identifying barriers in the tumor microenvironment

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Role for host-derived IL-12 and type 1 T cell response in anti-tumor immunity on mice

- IL-12 can promote superior CD8$^+$ effector T cell function (cytolytic activity and IFN-γ production) in vitro
- We found that tumor rejection correlated with an IFN-γ-producing T cell phenotype in tumor-draining lymph nodes in mice
- Host IL-12 also was required for spontaneous T cell response and tumor rejection (J. Immunol. 1996)
- Immunization with IL-12-transfected tumor cells could cause potent rejection of pre-established tumors (Int. Immunol. 1997)
- Argued for integration of IL-12 into tumor antigen-based vaccine approaches
Immunization with tumor antigen peptide-pulsed PBMC + IL-12 induces tumor protection
Induction of specific CTL responses in the blood of mice using tumor antigen peptide-pulsed PBMC + IL-12

Phase II trial of Melan-A peptide-pulsed PBMC + rhIL-12 (4 mcg):
Brief eligibility

- No clinically significant autoimmune disorders
- No immunosuppressive therapy
- HLA-A2+
- Melan-A expression by RT-PCR of tumor Bx
- Stable CNS metastasis allowed
- Prior therapies allowed
- Good performance status, acceptable hematopoietic and organ function, and life expectancy > 12 weeks
Phase II trial: Clinical outcome

<table>
<thead>
<tr>
<th>Best Response</th>
<th>Patients (n=20)</th>
<th>Number (%)</th>
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<tbody>
<tr>
<td>Complete response</td>
<td>2 (10)</td>
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<tr>
<td>Partial response</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Minor/mixed</td>
<td>5 (25)</td>
<td></td>
</tr>
<tr>
<td>Stable disease</td>
<td>4 (20)</td>
<td></td>
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<tr>
<td>Progressive disease</td>
<td>9 (45)</td>
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Phase II trial:
Greater increase in Melan-A-specific CD8⁺ T cells in clinical responders

Additional observations made in this phase II trial

• Some patients who progressed were found to have antigen-negative tumors grow out
  – Argues for immunization against multiple antigens
  – 4 peptides used concurrently in current phase II trial

• Some patients had a high T cell response even pre-treatment yet did not show tumor regression
  – Argues for downstream resistance at level of tumor microenvironment ➔ need to monitor tumor sites
Anti-tumor immune responses:
Taking into account the effector phase

Lymph node (Priming phase)
- APC
- nCD8
- IL-2

Blood
- eCD8

Lymphatic
- Vaccine
- Endogenous

Tumor microenvironment (Effector phase)
- APC
- IFN-γ
- Chemokines
- Inhibitory mechanisms
- Granzymes
- perforin

Inhibitory mechanisms
Recruitment of activated T cells to malignant ascites

Blood vs. ascites

Cytokine array: ascites

Tetramers: ascites

Also found expression of negative regulatory transcripts (PD-L1, FoxP3, IDO) and anergic T cells

Hierarchical clustering of metastatic melanoma biopsy gene array data: Main points

• 3 subsets defined ➔ Not all melanoma metastases are the same
• Immune transcripts dominate
  - T cell markers
  - Chemokines
  - Presence of negative regulators
• Group 3 “bland”, suggesting little inflammation
T cell transcripts are associated with expression of specific chemokine genes

Harlin et al. Manuscript submitted
Chemokine transcripts expressed in melanoma metastases in distinct tumor groups

• Group 1
  – CCL2/MCP1, CCL3/MIP1\(\alpha\), CCL4/MIP1\(\beta\),
    CCL5/RANTES, CCL19, CCL21/SLC
  – CXCL9/Mig, CXCL10/IP10, CXCL11, CXCL13
  – CXCL8/IL-8, CXCL12/SDF1

• Group 2
  – CXCL14
  – CXCL8/IL-8, CXCL12/SDF1

• Group 3
  – CXCL8/IL-8, CXCL12/SDF1
Human CD8$^+$ effector T cells can migrate to each of these key chemokines in vitro.
How to promote better recruitment?
Intratumoral LIGHT adenovirus in B16 melanoma

CD8+ T cell infiltrate

1st tumor
Ad-LIGHT
17.2%
Ad-control
4.56%

2nd tumor
Ad-LIGHT
14.9%
Ad-control
3.52%

Tumor rejection

Days after tumor challenge

Mechanisms of negative regulation of T cell function within the melanoma microenvironment

Highest in tumors that contain CD8+ T cells

1. Indoleamine-2,3-dioxygenase (IDO→ tryptophan catabolism)
2. PD-L1 (inhibitory ligand expressed by tumor cells)
3. CD4+CD25+FoxP3+ Tregs (extrinsic suppression)
4. T cell anergy (deficient B7 costimulation)
Group 1 (T cell-rich) is characterized by IDO and Group 2 (inflamed but T cell-poor) expresses Arginase
Co-expression of IDO, PD-L1, and FoxP3 transcripts in individual tumors
IHC for IDO, FoxP3, and PD-L1 shows expression in distinct cell subsets in melanoma metastases.

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<table>
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<tbody>
<tr>
<td><strong>A:</strong> IDO</td>
<td><strong>B:</strong> FoxP3</td>
</tr>
<tr>
<td><img src="image1.png" alt="IDO Image" /></td>
<td><img src="image2.png" alt="FoxP3 Image" /></td>
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<tr>
<td><strong>C:</strong> PD-L1</td>
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<tr>
<td><img src="image3.png" alt="PD-L1 Image" /></td>
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Metastatic melanoma tumors are B7-poor and can contain anergic T cells

![Graph of B7-1 expression in melanoma tumor gene array]

![ELISpot against autologous tumor cells]

**Stimulation:**
- T2
- T2/EBV
- Tumor

**EBV-specific Tumor-specific**
Preclinical studies have validated importance of each of these inhibitory mechanisms in the tumor context

- IDO
- PD-L1
- CD4⁺CD25⁺FoxP3⁺ Tregs
  - Anti-CD25 mAb, ex vivo bead depletion (Jones et al. Cancer Immun. 2002; Kline et al., submitted)
- Anergy
  - Intratumoral B7 (Bai et al. J. Immunol. 2001)
  - T cell-intrinsic anergy factors (diacylglycerol kinase—Zha et al, Nature Immunology, 2006)
- Combinations of negative regulatory pathway blockade
  - Synergy between Treg depletion and anergy reversal with homeostatic proliferation (Kline et al., submitted)
- These pathways are ready for targeting in the clinic
Human CD8+ effector T cells express CCL22/MDC

Control

CD3/CD28 6 days

ELISA

Chemokine concentration (pg/ml)

CCL3
CCL22

Media  Day 1  Day 5  Day 7

CD3/CD28
Supernatant from activated human CD8+ T cells recruits sorted CD4+CD25+ T cells in a CCL22-dependent fashion.
Can we profile tumor microenvironment and predict clinical outcome?

Current phase II study with multi-peptide vaccine

- 19 HLA-A2+ patients with metastatic melanoma
- All vaccinated with 4 peptides (MelanA, NA17, gp100, MAGE3) pulsed onto PBMC + rhIL12 q 3 weeks
- Patients had pre-treatment biopsy to verify expression of 2 antigens and prepare RNA for gene array analysis
- Clinically, 1 patient had a CR, 1 MR, and 4 had prolonged disease stabilization (6 months)
- Affymetrix gene array on pretreatment samples:
  - U133A chips utilized, data were normalized
  - Supervised hierarchical clustering done comparing patients with SD or better versus patients with PD
  - Looking for genes differentially expressed 2-fold or greater (low threshold)
Affymetrix gene array analysis of pre-treatment biopsies from patients on melanoma vaccine sorted by clinical outcome

Represents only 7 genes:
• 4 upregulated
• 3 downregulated

6 mos SD or better

Has implications for patient selection on vaccine trials, and understanding biology
Tumors from favorable clinical outcome patients express higher levels of TCRα, CXCL9, and CCL21
Tracking each step of the anti-tumor immune response longitudinally in patients
1. Potent T cell response induced by immunization with 4 peptides/PBMC/rhIL-12

Pre vs post

Kinetics
2. Increased CD8 transcripts in tumor post-vaccination

In addition, gene expression profile showed presence of chemokines and absence of IDO
3. Tumor regression, leaving behind pigment-laden macrophages
Summary of tumor microenvironment barriers:
Need to promote T cell trafficking and overcome local immunosuppression

- eCD8
- DC/Mφ
- IDO
- Arginase/iNOS
- PD-L1
- Tryptophan
- Arginine
- Granules
- Chemokines
- Cytokines
- Treg
- Anergy
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Melanoma vaccine trials
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PD-1/PD-L1
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Amy Peterson
Yuan-yuan Zha

Host requirements for anti-tumor immunity
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Hans Schreiber
Mike Spiotto

Human TIL analysis
Ruth Meng
Helena Harlin

T cell signaling
Sujit Janardhan
James O’Keefe
Candace Cham

Anergy and Tregs
Ian Brown
Justin Kline
Harald Wouters
John Strickler
Sujit Janardhan
Allen Ho
Reinhard Marks

Functional genomics core

Melanoma gene array
Helena Harlin
Amy Peterson
Mark McKee
Craig Slingluff

CD8 T cell metabolism
Candace Cham

Human TIL analysis
Ruth Meng
Helena Harlin

T cell signaling
Sujit Janardhan
James O’Keefe
Candace Cham
Yuan-yuan Zha
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   Sharmila Manoj
cGMP facility
Ramila Shah, Jessica Peyton
What can we do to overcome these barriers clinically?

1. Poor T cell trafficking
   - Intratumoral targeting of chemokines (or LIGHT)

2. Suppressive mechanisms
   - IDO inhibition
     - Small molecule inhibitors (1-MT)
   - PD-L1/PD-1 blockade
     - Monoclonal antibodies
   - Treg inhibition
     - Depletion in vivo/ex vivo
   - Anergy uncoupling
     - Intratumoral B7, homeostatic proliferation, small molecule inhibitors of anergy factors
Strategies to promote chemokine-mediated recruitment in tumor microenvironment

- Most straightforward for proof of concept is gene transfer approach
- One consideration is individual chemokines, but what if combination of several is important?
- Selection will be guided by ongoing mechanistic experiments
- But attractive alternative is LIGHT
  - TNF SF member that binds LTβR and promotes lymphoid architecture
  - Secondarily induces production of multiple chemokines by stroma
  - Has been shown to promote potent anti-tumor immunity when transfected into “difficult” tumors (Fu and colleagues)
What can we do to overcome these barriers clinically?

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     - Depletion in vivo/ex vivo
   - Anergy uncoupling
     - Intratumoral B7, homeostatic proliferation, small molecule inhibitors of anergy factors
1-methyltryptophan reverses immunosuppression by IDO and improves tumor control in vivo

PD-1<sup>−/−</sup> 2C TCR Tg T cells are superior at tumor rejection in vivo

![Graph showing tumor rejection](image)

Blank et al, Cancer Research, 2004
Ex vivo Treg depletion yields only partial tumor control following adoptive transfer into P14/RAG\(^{-/-}\) mice

Analysis of specific T cells ex vivo showed dysfunction → Treg depletion is not sufficient
Anergy reversal strategy #1: Homeostatic proliferation

Anergic 2C T cells reject tumors after homeostatic proliferation in RAG2\(^{-/-}\) hosts

Brown et al., J. Immunol., 2006
Anergy reversal strategy #2:
Inhibition of diacylglycerol kinase

Transduction to express DGK-α is sufficient to block RasGRP recruitment to T cell/APC interface

Also blocks IL-2 production and MAPK activation
A pharmacologic inhibitor of DGK recovers IL-2 production by anergic Th1 cells

DGK is thus a negative regulator of T cell function in anergic state amenable to pharmacologic manipulation
Combinatorial approaches to counter multiple negative regulatory features in concert

- Our human melanoma metastasis analysis suggests that at least 4 inhibitory mechanisms are present in tumors that contain CD8\(^+\) T cells
- It may be necessary to interfere with anergy, Tregs, PD-1, and IDO to achieve maximal therapeutic benefit from activated tumor antigen-specific CTL
- First experiments studying Treg depletion in combination with anergy reversal through HP
Combined Treg depletion and anergy reversal through homeostatic proliferation supports rejection of B16 melanoma and leads to vitiligo

Kline et al., submitted
CD25 depletion plus homeostatic proliferation improves the effector function of tumor-specific T cells
New clinical protocols to counter inhibitory mechanisms

- Depletion of Tregs using Ontak prior to vaccination with 4 melanoma peptides (Trial recently opened)
- Preparation of CD25-depleted T cell product for adoptive transfer back into sublethally irradiated patient (protocol submitted to IRB)

- Medarex developing anti-PD-1 mAb for clinical testing
- RAID program preparing clinical grade 1-MT for testing IDO inhibition in patients

- Anti-CTLA-4 mAbs already in phase III trials by Pfizer, BMS (first proof of concept to uncouple negative regulation)
1. PD-1/PD-L1

- PD-1: receptor induced on activated T cells
- Contains ITIM and ITSM domains that can recruit SHP2
- PD-1-deficient mice develop autoimmune syndromes => dominant role is negative
- Two defined ligands: PD-L1/B7-H1 and PD-L2/B7-DC
- PD-L1 can be expressed in non-hematopoietic tissues, including tumor cells
- PD-L1 expression is upregulated by IFN-γ
IFN-γ upregulates PD-L1 on all human melanoma cell lines tested
PD-L1 protein is present in metastatic melanoma deposits

Control Ig  Anti-PD-L1
Elimination of PD-L1/PD-1 interactions enables T cell activation by IFN-γ-treated B16.SIY melanoma.
2. T cell anergy

- Can result from TCR ligation in the absence of CD28 costimulation by B7-1/B7-2
- Characterized by defective TCR-induced cytokine production and proliferation
- Defined by blunted TCR-induced Ras activation
  - (Fields, Gajewski & Fitch Science 1996)
- Hypothesized to represent one mechanism of tolerance to antigens expressed by tumors
- Reversible by proliferation via cytokines that engage the c\(\gamma\) chain (e.g. IL-7)
Dysfunction of 2C TCR Tg T cells isolated from P1.HTR tumor-bearing P14/RAG2-/- mice consistent with anergy.
Strategies to prevent and/or reverse tumor-induced T cell anergy

1. Dissect biochemical mechanism and develop pharmacologic strategy to restore function (i.e. manipulate the T cell)
2. Reverse through proliferation by homeostatic cytokines (i.e. manipulate the host environment)
3. Transfer expression of B7-1 into established tumor sites (i.e. manipulate the tumor)
Strategy 1:
New anergy target diacylglycerol kinase

Zha et al Nature Immunol. 2006
Transduction to express DGK-α is sufficient to block RasGRP recruitment to T cell/APC interface

Also blocks IL-2 production and MAPK activation
A pharmacologic inhibitor of DGK recovers IL-2 production by anergic Th1 cells

DGK is thus a negative regulator of T cell function in anergic state amenable to pharmacologic manipulation
Strategy 2: Homeostatic proliferation
Anergic T cells partially recover function in RAG2\(^{-/-}\) recipients

A: Homeostatic proliferation

B: IL-2 recovery
3. Regulatory T cells

- Defined by CD4⁺CD25⁺ phenotype
- Selectively express the transcription factor FoxP3, and preferentially express the TNFR family member GITR
- Functionally suppress activation of CD4⁺ and CD8⁺ effector T cells in vitro and in vivo
- Observed to be present in increased numbers in cancer patients and within tumors
Tumor-infiltrating human melanoma metastases can contain $\text{CD4}^+\text{CD25}^+\text{FoxP3}^+$ T cells

These suppress activation of conventional T cells ex vivo
Tregs are expanded in mice bearing B16 melanoma

GFP-FoxP3 knock-in mice
4. Indoleamine-2,3-dioxygenase (IDO)

- Catabolizes tryptophan, an essential amino acid
- Expressed in placenta, but also in cells in tumor microenvironment
- Induced by IFN-γ
- Leads to T cell hypo responsiveness and apoptosis
- Inhibitor, 1-methyl-L-tryptophan, can potentiate anti-tumor immunity in mice
Presence of multiple negative regulatory mechanisms may require a combinatorial approach for therapy.
The issue at hand:
Why have cancer vaccines shown limited efficacy in patients with metastatic melanoma?

- Multiple immunization approaches have been investigated in clinical trials with 1000s of patients
- Increased frequency of tumor antigen-specific T cells have been detected in the peripheral blood in response to vaccines
- In many cases these are functional (IFN-γ-producing, cytolytic) when analyzed ex vivo from the blood
- In addition, many patients have spontaneous immune responses detected in the blood against tumor antigens even without immunization
- Despite these achievements, clinical response rates have been low
Clinical outcome of all melanoma patients treated on phase I and phase II clinical trials of melanoma peptide vaccine + IL-12 at Chicago

n=55
ORR ~ 12%, 3 CRs
MS=298 days
Still, 23% alive at 2 years
Schema of phase I study of MelanA or MAGE-3 peptide-pulsed PBMC + rhIL-12 in patients with metastatic melanoma

- **SCHEMA:**
  - HLA-A2+
  - Biopsy+ for MelanA or MAGE-3

- **VACCINE**
  - PBMC+ MAGE-3 or MelanA

- **IL-12 DOSE**
  - 0
  - 30
  - 100
  - 300 ng/kg
  - Day 1
  - Days 1, 3, 5

- **ENDPOINTS:**
  1. Toxicity
  2. CD8+ T cell IFN-γ production
  3. Clinical response/survival

- **Cycles repeated every 3 weeks**
- **Re-evaluation every 3 cycles**
Phase I vaccine trial: Conclusions

• Immune responses could be induced in advanced melanoma patients
• Intermediate doses of rhIL-12 (30-100 ng/kg) appeared to be optimal
• Patients with elevated serum LDH had no clinical benefit

Treatment Schema

ELISPOT: 3 weeks 3 weeks 3 weeks

Peptide/PBMC:

rhIL-12 (4 µg):
Recognition of class I MHC-restricted tumor antigen peptides by CD8⁺ CTL

• Most tumors express antigens that can be recognized by CD8⁺ CTL
• First human tumor antigen gene cloned in 1991 by Thierry Boon from melanoma
• Made it possible to analyze immune responses in patients, and also to develop antigen-specific vaccines

Questions for us in 1995-1997:
• How best to immunize?
• Will immunization be sufficient?
Barrier #1: T cell trafficking
Immunohistochemistry for CD8, CD20, and CD68 in T cell “high” versus T cell “low” tumors

**CD8**
(T cells)

**CD20**
(B cells)

**CD68**
(Macrophages)

T cells: High | Low
Some chemokine mRNAs are preferentially expressed in tumors with T cell infiltrates and some are expressed broadly.
Differential chemokine expression in solid tumor lysates as detected by protein array

High T cell
- IL-8
- MCP1/CCL2
- MIP1β/CCL4
- RANTES/CCL5

Low T cell
- 38 chemokines represented

% of positive control

% T cell lower  % T cell higher
Pt. groups

CCL5  CCL2  CCL4  CCL18
Differential chemokine expression in melanoma metastases with high versus low T cell transcripts
Chemokine receptors expressed on CD8+ effector T cells by gene array analysis and confirmatory FACS

<table>
<thead>
<tr>
<th>Receptor:</th>
<th>Defined chemokines that bind:</th>
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<tbody>
<tr>
<td>CCR1</td>
<td>MIP-1α, RANTES, MCP-3, HCC-1</td>
</tr>
<tr>
<td>CCR2</td>
<td>MCP-1, MCP-3</td>
</tr>
<tr>
<td>CCR5</td>
<td>MIP-1α, MIP-1β, RANTES</td>
</tr>
<tr>
<td>CXCR3</td>
<td>Mig, IP-10, I-TAC</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Also expressed on naïve T cells, but</td>
</tr>
<tr>
<td></td>
<td>still may be relevant in effector cells</td>
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<tr>
<td>CCR7</td>
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Rare melanoma cell lines express a broad array of chemokines

- Implies that in some cases, the melanoma tumor cells themselves can produce the entire panel of key chemokines
- Which chemokines are necessary to support recruitment of CD8+ effector T cells?
Combined chemokine blockade of supernatant from melanoma cell line 537 prevents migration of CD8^+ effector T cells in vitro.
Barrier #2:
Local immunosuppression
Real-time RT-PCR analysis confirms reciprocal expression of IDO and Arginase in individual tumors

![Graph showing reciprocal expression of IDO and Arginase](image)