CD39 Expression by Regulatory T and NK(T) Cells: Immune Suppression in Tumors

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Updated Conflicts and Disclosures

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ATP, ADP and other nucleotides/sides: thromboregulatory purinergic agonists for platelets and vascular endothelial cells

Links with immune responses...
T regulatory and NKT cells

Involvement of purinergic signaling and CD39 (E-NTPDase1) ectonucleotidase in cancer....
Purinergic Mechanisms

Einstein: “Make everything as simple as possible, but not not simpler”.

>1 billion years of directed evolutionary responses and adaptations...integration mechanisms of thrombosis and immunity
Mechanisms of ATP release

- Exocytosis
- Transporter, Porin?
- Connexin, Hemichannels
Membrane Bound P2 Receptors

LIGAND-GATED ION CHANNEL
P2X1 – P2X7

G-PROTEIN COUPLED RECEPTOR
- TO PHOSPHOLIPASE C
  [P2Y11 AND P2Y12 ALSO TO ADENYLATE CYCLASE]
- Gq / Gi/o
CD39/Ecto-Nucleoside Triphosphate Diphosphohydrolase (E-NTPDase) Ectonucleotidases

1. Identity of vascular ecto-ADPases shown to be CD39 (and other family members exist to E-NTPDase)

2. Ectonucleotidase cascade in tandem with CD73/ecto-5’-nucleotidase

3. CD39 is dominant vascular (and immune) ectonucleotidase

4. Much published work in thromboregulation in vasculature

Cardiac arteriole:
CD39 (green) for EC, SM actin (blue) and CD39L1 (red) for pericytes
Adenosine

CD39

1: Adenosine generation
Platelet de-aggregation
Endothelial cell protection

CD73

2: Scavenges nucleotides

3: Prevents desensitization

Endothelial cell

Thromboregulatory roles for CD39
Immunity and Coagulation

Integration of mediators that drive inflammation, platelet activation and immune reactions...

Coagulation Platelets

Purinergic signals via nucleotides

Inflammation

Cellular Immunity
CD39 expression by T cells

- T regulatory cells modulate vascular injury
- Adoptive transfer of CD39 transgenic marrow has profound effects on hemostasis and vascular inflammatory insults in reperfusion injury in mice
- CD39 expression patterns on T cells? ....as sources of immune suppressive adenosine
T regulatory cells have lacked reliable surface markers

Adapted: Wing et al. Scand J Immunol 2005
CD39 is a good surface marker of T regulatory cells

a) Phenotype

b) Gene profile

- CD39
- CD25
- IL-10
- foxp3
- GITR
- CTLA4

- CD4+/CD39+
- CD4+/CD39-

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Relative gene expression

Suppressive Function

Cpm x 10^-3
Regulatory T cells co-express CD39 and CD73

a) Phenotype

Total cells from lymph nodes

Sorted GFP*(Foxp3*)

CD39-PE

CD39-PE

b) Gene profile

Relative gene expression

Fo xp3

GITR

Cpm x 10^-3

c) Suppressive Function

Effector T cells

Foxp3+/CD39+/CD73+
Adenosine is the final product of CD39 and CD73 activity

\[
\text{ATP/ADP} \xrightarrow{\text{CD39}} \text{AMP} \xrightarrow{\text{CD73}} \text{ADENOSINE}
\]
CD39⁺/CD73⁺ Treg can generate adenosine
CD39 generated adenosine suppresses T cell responses via cellular A2A receptors

Relative A2a expression

Resting Activated Resting Activated

CD4+CD25- T lymphocytes

AC: accessory cells

A2A agonist ATL146e

Cpm x 10^-3

Day 2 Day 4 Day 6

A2A null

*: p=0.002

Cd39 null

Cd39 null + NTPDase

*: p=0.002
Vascular injury, organ grafts and T regulatory cells

- Cytopathic/effector
- Regulatory

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REJECTION

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TOLERANCE
Treg from \( Cd39 \) null mice fail to suppress Teff responses in vivo.

\( cd39 \) null

or

WT C57BL/6

\( \text{CD}^+\text{CD}25^+ \)

\( \text{CD}^+\text{CD}25^- \)

SKIN REJECTION

\( \% \text{allogeneic skin survival} \)

\( \text{Time (days)} \)

\( p=0.02 \)

- null \( \text{CD}^+\text{CD}25^- \)
- WT \( \text{CD}^+\text{CD}25^- \)

\( n=9 \)

\( n=8 \)

\( n=6 \)

\( n=6 \)
Molecular model of T regulatory “adenosinergic suppression”

**T regulatory cell**
- CD4
- CD25
- TCR
- CD73
- CD39
- Foxp3

**Adenosine-2A Receptor**
- ATP/ADP
- ADENOSINE

**ADO drives Treg**
- [↑cAMP]
- Inhibition of tissue inflammation and injury

**T effector cell**
- CD4
- TCR

*Deaglio, Dwyer and Gao, J. Exp. Med 2007*
Nucleotides as proinflammatory cytokines and involvement in generation of Th17 cells

- Th17 cells are an independent subset that plays a role in protection against extracellular pathogens (bacterial and fungal infections), cancer.

- Th17 cells are highly proinflammatory and mediate severe autoimmunity...appear selected in Cd39 null mice.

- Proposed that ATP drives Th17...and Ado impacts Treg and NK(T)...??
• Hepatic metastatic disease remains a major clinical problem and novel therapeutic options are required

• Melanoma is an aggressive tumor that often targets the liver

• The incidence of this tumor is increasing by an additional 5-7% per annum and the prognosis for metastatic disease remains dismal

• Appreciation of the cellular mechanisms involved in metastatic melanoma may help to improve cancer therapy
Deletion of vascular endothelial CD39 inhibits angiogenesis, transplanted tumor growth and liver regeneration

**Matrigel Invasion**

*Wild-type*  
*Cd39-null*

**Tumor Growth**

Murine model: metastatic liver cancer

- Metastatic cancer in liver was modeled via portal vein infusion of luciferase-expressing melanoma B16/F10 cells (luc-B16/F10, $1.5 \times 10^5$)

- Imaging of tumor size was done in vivo by bioimaging system IVIS 50 (Xenogen)

- Tumor volume was determined by integration: $S_1 + t_2 + \ldots + t_n$
  \[ t = a^2 \times b \times 0.52; \ a = \text{smaller tumor diameter, } b = \text{the larger tumor diameter} \]
Deletion of Cd39 inhibits growth of hepatic metastases
Cd39 null BM-derived cells inhibit tumor growth
Immunohistochemical images of mouse livers (d10)

<table>
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<th>CD39</th>
<th>CD31</th>
<th>Tunel</th>
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<tr>
<td>nullBM-null</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
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Characterization of infiltrating BM-derived cells

Immunohistochemistry (wt-null)

- Thy1.2
- CD4
- MΦ
- CD8
- Gr-1
- CD39
- CD11b
- Foxp3 (X400)

FACS

- CD39
- CD11b
- Gr-1
- TCRβ

Gated on NK1.1+ cells
Low ADPase activity of Cd39 null tumor infiltrating lymphocytes

Tumor infiltrating lymphocytes

<table>
<thead>
<tr>
<th></th>
<th>Std</th>
<th>WT</th>
<th>Cd39 null</th>
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<tr>
<td>(min)</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>15</td>
<td>15</td>
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</table>

- Adenosine
- AMP
- ADP
Adoptive transfer of cells to Rag1 null mice

Wild type Treg cells (*CD4+GFP+, 0.1 X 10^6)
and/or
Teff cells (*CD4+GFP-, 0.9 X 10^6)
Freshly isolated on MOFLO

Luc-B16/F10 (2 X 10^5)

* Obtained from spleen and LN of Foxp3-GFP knockin mice
Wild type (CD39+CD4+Foxp3+) Tregs suppress NK cell proliferation and IFN gamma secretion

Adoptive transfer in rag1-/- mice

Absolute NK cell No. (x 10^3)

Treg  Teff  Treg+Teff

p=0.039

Serum IFN-γ

pg/ml

Treg  Teff  Treg+Teff
Heightened proliferation of endogenous liver NK cells was observed in rag1-/-- mice transferred with Cd39 null CD4+ cells.
Adoptive transfer to rag2/γc d -/- mice

CD4⁺ T cells (1 X 10⁶, wt and Cd39 null) and/or CD8⁺ T cells (0.5 X 10⁶, wt) NK cells (varied, wt and Cd39 null). Freshly isolated on MOFLO

i.v.  
day, -1  

rag2/γc d -/-

luc-B16/F10 (2 X 10⁵)

p.v.  
day, 0  

rag2/γc d -/-

day, 14  

Sacrifice
Examine tumor
Collect serum
Harvest tumor tissues
Isolate liver MNC
Analyze by FACS
NK cells are responsible, at least in part, for inhibition of tumor growth.

**Graph 1:**
- **Graph Title:** Tumor volume (cm³)
- **Legend:**
  - rag1/−/−
  - rag2/yc d −/−
- **Statistical Significance:** p=0.021
- **Data Points:**
  - rag1/−/−: Tumor volume ≈ 1 cm³
  - rag2/yc d −/−: Tumor volume ≈ 2 cm³
- **Label:** n=5 per group

**Graph 2:**
- **Graph Title:** Tumor volume (cm³)
- **Legend:**
  - wtCD4
  - nullCD4
  - wtCD4+wtCD8
  - nullCD4+wtCD8
- **Data Points:**
  - wtCD4: Tumor volume ≈ 2.5 cm³
  - nullCD4: Tumor volume ≈ 2.5 cm³
  - wtCD4+wtCD8: Tumor volume ≈ 3 cm³
  - nullCD4+wtCD8: Tumor volume ≈ 3 cm³
- **Label:** n=5 per group
Reconstitution with NK cells decreased tumor growth in rag2/γc d -/- mice

**Tumor volume (cm$^3$)**

- **rag2/γc d -/-**
- **rag2/γc d -/- plus NK**

**% of NK cells in liver MNC**

- **rag2/γc d -/-**
- **rag2/γc d -/- plus NK**

$p<0.001$

$n=6$ per group
Adoptive transfers to NK deficient Rag2/γc double null mice

Wt or *Cd39 null Treg cells (1 X 10^6)
Co-transfer with NK cells (1 X 10^6)

Luc-B16/F10 (2 X 10^5)

* Obtained from spleen and LN of Foxp3-GFP knockin/Cd39 knockout mice. Foxp3-GFP knockin mice and Cd39-/− mice were crossed to create Foxp3-GFP knockin/Cd39 knockout mice.
Cd39 null (CD39-CD4+Foxp3+) Tregs fail to suppress NK cell-mediated effects
CD39 on NKT (NK1.1+, CD3+)

Wild type

CD39 null

Beldi et al Hepatology 2008
Expression of CD73 by NKT cells

Liver MNC

NK
NKT
Treg cells

Expression of CD73 by NKT cells

CD73
Polyoxometalates (POM) and NTPDases

- Polyoxometalates (POMs) are metal complexes previously proposed as chemotherapeutic agents in human cancers.
- These compounds contain transition metal ions, e.g. tungsten, molybdenum, vanadium, bridged by oxygen atoms.
- These drugs block NTPDase activity - inhibition of CD39 using POM-1 precludes renal vascular protection by ischemic preconditioning.

Pharmacological blockade of CD39 activity using POM-1 abrogates tumor growth

WT mice treated with POM

<table>
<thead>
<tr>
<th>0 mg/kg/day</th>
<th>5 mg/kg/day</th>
<th>10 mg/kg/day</th>
<th>20 mg/kg/day</th>
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</thead>
<tbody>
<tr>
<td>Tumor volume (cm³)</td>
<td>p&lt;0.001</td>
<td>*</td>
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</table>

*n=6 per group

* Mice have comparable liver and renal function, compared to control mice
Conclusions

• CD39 is highly expressed by T regulatory (and NKT) cells, and modulates immune reactions in vivo

• Nucleotides/nucleosides impacting platelet activation may also have major effects on cellular inflammatory reactions and/or immune responses important in cancer
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Laboratory Researchers...
POM-1 blocks NTPDase activity of CD39 on Tregs in vitro; using TLC analysis

Wild type Tregs (CD39+CD4+Foxp3+, $3 \times 10^5$) were pre-treated with POM-1 (26 μm) at 37°C for 30 min and subjected to TLC analysis
Cd39 null lymphocytes from tumor DLN (draining lymph nodes) demonstrate elevated cytotoxicity to melanoma B16/F10 cells in vitro.

The cytolytic activity of purified lymphocytes from DLN (A) and spleen (B) of tumor-bearing wild type and null mice is tested against tumor target B16/F10 cells at a E:T ratio of 64:1. The viability of remaining B16/F10 cells is examined using MTT assay kit following the manufacturer’s instructions (ATCC, Manassas, VA).
### Intratumor cytokine profiles of tumor-bearing mice at day 10

<table>
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<tr>
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<th>MCP-1</th>
<th>PDGF-BB</th>
<th>VEGF</th>
<th>IFN-γ</th>
<th>IL-2</th>
<th>IL-4</th>
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<tr>
<td>wt-wt</td>
<td>242±99</td>
<td>67±18</td>
<td>126±89</td>
<td>118±43</td>
<td>25±16</td>
<td>3.4±0.57</td>
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<tr>
<td>null-wt</td>
<td>145±14</td>
<td>42±9</td>
<td>76±25</td>
<td>123±45</td>
<td>16±3.8</td>
<td>6.5±2.69</td>
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<tr>
<td>wt-null</td>
<td>234±75</td>
<td>52±3</td>
<td>137±113</td>
<td>132±39</td>
<td>16±4.2</td>
<td>5.7±1.12</td>
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<td>null-null</td>
<td>90±35</td>
<td>46±13</td>
<td>28±6</td>
<td>105±8</td>
<td>17±4.5</td>
<td>8.4±2.59</td>
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<th>IL-10</th>
<th>IL-17</th>
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<th>TGF-β</th>
<th>MMP-9 (X10³)</th>
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<td>35.0±24.4</td>
<td>6.4±1.4</td>
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<td>2.69±0.9</td>
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<td>78±63.5</td>
<td>25.5±35</td>
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<td>31.2±24.5</td>
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<td>7.28±1.7</td>
<td>872±561</td>
<td>29.3±35.8</td>
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All data are standardized by protein concentration of tumor tissue homogenates (pg/ml/ug protein). Red indicates that the p value is <0.05 between wt-wt and null-null groups.