1. The invariant TCR expressed by iNKT cells recognizes a limited repertoire of glycolipids in the context of CD1d molecules expressed by DC and B cells.

2. iNKT cells are CD40L positive and the cross talk between iNKT cells with DC and B cells results in CD40 dependent DC maturation and B cell activation.

3. TLR signaling events up-regulate endogenous CD1d ligand(s), which in combination with IL-12 activate iNKT cells.
Melanoma
Reduced expansion of Melan-\(\text{A}_{26-35}\) specific T cells in total leukocytes

Ficoll purified PBMC  Total leukocytes

Melanoma patient

Healthy donor

Carmen De Santo
Arginase 1 expression by neutrophils from patients with melanoma

Healthy donor

Melanoma patient

ICS

Healthy donors  Melanoma patients

Arginase

GAPDH
Intracellular staining with anti IL-10 Ab of neutrophils from patients with melanoma

Rat IgG1

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Melanoma patient</th>
<th>Healthy donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>2.6%</td>
<td>0.7%</td>
</tr>
<tr>
<td>CD15</td>
<td>98%</td>
<td>0.93%</td>
</tr>
</tbody>
</table>

Healthy donor

Melanoma patient
IL-10 secretion by neutrophils from patients with melanoma
IL-10 production by neutrophils from patients with melanoma inhibits Melan-A\textsubscript{26-35} specific T cell response

- CD11b\textsuperscript{+}CD15\textsuperscript{+} cells
- IL-10R-blocking Ab
- CD11b\textsuperscript{+}CD15\textsuperscript{+} cells
+ IL10R-blocking Ab

<table>
<thead>
<tr>
<th></th>
<th>- CD11b\textsuperscript{+}CD15\textsuperscript{+} cells</th>
<th>+ CD11b\textsuperscript{+}CD15\textsuperscript{+} cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.4%</td>
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</tbody>
</table>
Frequency of neutrophils in patients with melanoma correlates with staging of disease and their suppressive activity.
Cytokine concentration in the plasma of patients with melanoma
Plasma levels of Serum Amyloid A (SAA) in patients with melanoma correlates with disease staging.

De Santo et al., Nature Immunology (in press)
Correlation between frequency of CD11b+CD15+ cells and SAA plasma levels
SAA production by TAM and melanoma cells

unlab

anti CD68

SAA

SAA/CD68
Serum Amyloid A

- SAA: 12Kd glycoprotein mainly secreted by hepatocytes during acute and chronic inflammation. It has also been shown to be secreted by pneumocytes and macrophages

- SAA secretion is induced by IL-6, IL-1β, TNF α, LPS

- Described activities: 1) chemoattractant, 2) induces G-CSF secretion and neutrophilia, 3) enhances ROS production, 4) opsonization of bacteria by binding to OmpA family members on G- bacteria

Does incubation of neutrophils with SAA induce IL-10 secretion?
SAA binding receptors

FPR-2

TLR-2

CD38

NAD^+

Ca^2+

cADPR

RyR

Ca^2+

MEKK

MEK3

MEK6

p38-MAPK

RAS

RAF

MEK1

MEK2

ERK1

ERK2

Gβ

Gα

Gγ

PLC

PIP2

IP3-K

IP3

DAG

PKC

CaM-K

Calmodulin

Ca^2+
Neutrophils express both FPR2 and TLR2
Incubation of neutrophils from healthy donors with SAA induces IL-10 secretion.
SAA binding to FPR-2 controls IL-10 secretion

SAA-1

Anti-FFPR2

Anti-TLR2

FMPL

Pam3Cys

IL-10 (ng/ml)
Conclusions (I)

A large proportion of primary melanoma and TAM secrete SAA

SAA controls the differentiation of immunosuppressive IL-10 secreting neutrophils

IL-10 secretion from SAA treated neutrophils is FPR-2 dependent

SAA levels in plasma of patients with melanoma correlates with staging of disease and frequency of IL-10 secreting neutrophils

Melanomas exploit of a physiological role of SAA

High SAA levels in melanoma patients should be considered a novel tumor evasion mechanism to differentiate IL-10 producing neutrophils

Future vaccine clinical trials in patients with melanoma should combine strategies to reduce IL-10 immunosuppressive activity
Neutrophils are CD1d and CD40 positive
Cross-talk between neutrophils and iNKT cells

SAA

- from healthy donors
- from melanoma patients

iNKT cell line

Adaptive Immune Response
Neutrophils from healthy donors pre-treated with SAA activate iNKT cells.
Neutrophils purified from melanoma patients directly activate iNKT cells

![Flow cytometry image](image)

<table>
<thead>
<tr>
<th>Condition</th>
<th>IFN-γ (copies/mL)</th>
<th>CD1d-tetramer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>SAA</td>
<td>79.0</td>
<td></td>
</tr>
<tr>
<td>αGalCer</td>
<td>80.0</td>
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<tr>
<td>Anti CD1d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>blocking Ab</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: The figure shows flow cytometry results comparing untreated, SAA-treated, and αGalCer-treated samples, with antibody blocking of CD1d.
Cross-talk between innate and adaptive immune system to optimize vaccination strategies

- Neutrophils
- iNKT cell line

Adaptive Immune Response
Reduction of IL-10 secretion after incubating neutrophils from melanoma patients with iNKT cells

- iNKT cells
+ iNKT cells
Blocking anti-CD1d and iNKT cells
Blocking anti-CD40 and iNKT cells

IL-10 (ng/ml)

CD11b^+CD15^+ cells
Reduction of IL-10 secretion from SAA treated neutrophils is dependent on the numbers of iNKT cells.
Harnessing iNKT cells abolishes neutrophils’ suppressive activity and restores Melan-A\textsubscript{26-35} specific CD8\(^+\) T cell response.

\(\alpha\)-GalCer pulsed CD11b\(^+\)CD15\(^+\) cells

+ Autologous iNKT cells

+ CD1d-blocking Ab + CD40-blocking Ab

Soluble CD40L

\begin{tabular}{c|c|c|c|c}
0.02\% & 6\% & 0.04\% & 0.05\% & 8\%
\end{tabular}
Reduction of IL-10 secretion from SAA treated neutrophils is CD40 dependent.

Graphs showing the phosphorylation levels of ERK, p38, and Akt in untreated and CD40L treated samples.

Bar graph showing IL-10 secretion levels in the presence and absence of CD40L at different doses.
SAA treated neutrophils can interact with iNKT cells

1. FPR-2
2. CD1d

Melanoma Patients

SAA-1

IL-10

IL-12

CD40

γ-IFN

TCR

CD40 L

γ-IFN
CD1d<sup>+/+</sup>/CD1d<sup>−/−</sup> mixed bone marrow chimera mice

- CD45.1<sup>+</sup> (SJL)
- CD1d<sup>+</sup> CD45.1<sup>+</sup>
- CD1d<sup>−</sup> CD45.2<sup>+</sup>
- CD1d<sup>−</sup> CD45.2<sup>+</sup>
- iNKT cells CD45.1<sup>+</sup>

SAA-1 injection s.c. (120µg/mouse)
5 daily injections

Analysis to 0, 5, 8 day

900 rads
6-8 wks
Injection of SAA-1 into CD1d mixed bone marrow chimeras results in the expansion of IL-10 neutrophils only from CD1d - neutrophils.
CD1d- IL-10 secreting neutrophils from CD1d +/-/CD1-/- chimera inhibit OT-I proliferation
Conclusions (II)

- Incubation of neutrophils with SAA facilitates their cross-talk with iNKT cells, which results in iNKT cell activation and reduced secretion of IL-10

- SAA dependent interaction between neutrophils and iNKT cells is CD1d and CD40 dependent

- Injection of SAA in CD1d\(^{+/+}\)/CD1d\(^{-/-}\) mixed bone marrow chimeras induces IL-10 secretion mainly from CD1d- neutrophils

Harnessing iNKT cells should be considered in melanoma patients to reduce the activity of immunosuppressive neutrophils
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