Azathioprine: Mechanism of action

Markus Neurath
IBD - Pathogenesis

Genetic predisposition

Bacterial Antigens

Activation of the Mucosal Immune System

Th1

Crohn’s disease

Ulcerative colitis

Th2

NK T

Colitis-associated colon carcinoma chromoendoscopy
T cells are key effector cells in IBD.

- **Crohn’s disease**: High IL-12, low EBI3
  - T helper 1
  - Bacterial Antigens
  - Dendritic Cells

- **Ulcerative colitis**: Low IL-12, high EBI3
  - T helper 2 (?)
T cell apoptosis and IBD

- In the antigen-rich environment of the gut T cell expansion is controlled by apoptosis

- LP T cells exhibit increased susceptibility to Fas-mediated apoptosis compared to blood T cells

- IBD T cells exhibit reduced apoptosis induced by IL-2 deprivation and Fas/FasL activation. 

- This T cell resistance to apoptosis contributes to T cell expansion and disease perpetuation in IBD

- Antibodies to proinflammatory cytokines appear to suppress intestinal inflammation by the induction of T cell apoptosis
Blockade of the IL-6/ sIL6R system induces apoptosis in Crohn’s disease

Atreya Nat. Med. 2000
Anti-TNF Antibodies induce apoptosis of LP CD4+ T cells in CD
TenHove et al. Gut 2002
Van den Brande et al. Gastro 2003
Azathioprine/ 6-Mercaptopurine

Discovered 6-mercaptopurine, thioguanine (JBC 1951) and azathioprine (Cancer Res. 1963)

Screening of 100 anti-metabolites for growth suppression of Lactobacillus casei

1988 Nobel Price in Physiology or Medicine

Gertrude B. Elion (*1918-1999)
Azathioprine/ 6-MP in IBD

1962 First case report on 6-MP in IBD by R.H.D. Bean

38 year old patient with chronic active UC

300mg 6-MP: reduction of blood transfusions, X-ray: return of haustration, endoscopy: healing of colonic ulcers

50 mg 6-MP: return of clinical symptoms

300 mg followed by 100 mg 6-MP: Long-term remission over 80 weeks
Induction of remission by azathioprine/ 6-MP in Crohn´s disease

**Azathioprine**
- Candy (1995)
- Ewe, Meyer zum Büschenfelde (1993)
- Klein (1974)
- Rhodes (1971)
- Summers (1979)
- Willoughby (1971)

**All azathioprine trials**

**6-Mercaptopurine**
- Oren (1997)
- Present (1980)

**All 6-MP trials**

**All trials**

\[
\begin{array}{c|c|c}
& Odds Ratio [95 % CI] & \\
\hline
Azathioprine & \\
Ewe, Meyer zum Büschenfelde (1993) & \\
Klein (1974) & \\
Rhodes (1971) & \\
Summers (1979) & \\
Willoughby (1971) & \\
\hline
6-Mercaptopurine & \\
Oren (1997) & 3,34 [1,67; 6,66] \\
Present (1980) & \\
\hline
\end{array}
\]

Pearson et al. 1995, Stange et al. 2002
How does azathioprine work?

This mechanism of action in IBD would lead to:
- random efficacy
- predominant effects in rapidly dividing cells (e.g. epithelial cells)
- random side effects
The antiproliferative effect requires high dosages of 6-MP

Relative cell number/ CFSE labeling

PBL cultured for 4 days

Clinical therapy of IBD and T cell apoptosis

Anti-TNF antibodies

Induction of T cell apoptosis (cell death)

Azathioprine/ Rac1

TNF-R2

Azathioprine induces T cell apoptosis

CD45RA cells

untreated

azathioprine

6-MP

CD45RO cells

untreated

azathioprine

6-MP
Dose-dependent effects of azathioprine

**6-TG levels:** 31.5 168 pmol/mg DNA

Levels in IBD patients under long-term therapy: 100-2305 pmol/mg DNA (Cuffari 1996)
6-MP effects in IBD patients

A

untreated

propidium iodide

annexin V

7%

6-MP

propidium iodide

annexin V

41%

B

apop. cells (%)

- + - + + culture
thephy

6-MP

IBD no 6-MP IBD 6-MP IBD 6-MP NR

C

D

E

F

G

H

IBD no 6-MP IBD 6-MP IBD 6-MP NR
Effects of 6-thioguanine on apoptosis

untreated

6-MP

6-TG

propidium iodide

annexin V

17%

37%

49%

Effects of 6-thioguanine on apoptosis
Selectivity of azathioprine for CD28 signaling

- **6-MP**
  - 73% induction of apoptosis
  - 41% NFκB activation

- **anti-CD3**
  - +: +
  - -: -

- **anti-CD3/28**
  - +: +
  - -: -

**Bcl-xL** levels:
- **untreated**
  - Counts: 50
- **6-MP**
  - Counts: 50
  - 41% activation

**Gene Expression**:
- **Bcl-xL**
- **IL2 R**
- **TACI**
- **GAPDH**

**6-MP concentrations**:
- 0, 5 µmol
6-MP induces a mitochondrial pathway of apoptosis

**caspase-8**

**caspase-9**

**CD45RA** **CD45RO**

**relative light units**

**6-MP**

0 5 0 5 µmol

**CD45RA** **CD45RO**

**relative light units**

**6-MP**

0 5 0 5 µmol

**membrane potential**

**FCCP**

counts

**membrane potential**

**azathioprine**

counts
6-MP induced apoptosis requires caspase-9

untreated

6-MP

6-MP + Ac-LEHD CHO

6-MP + Ac-IETD CHO
Azathioprine suppresses NF-kappaB activation

**EMSA: NF-kappaB**

NF-κB p50/65

<table>
<thead>
<tr>
<th>Jurkat</th>
<th>CD3</th>
<th>CD3/28</th>
<th>+6-MP</th>
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CD4+ T cells

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<tr>
<th>CD3/28</th>
<th>anti-p50</th>
<th>anti-p65</th>
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**EMSA: SP1**

SP1

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CD4+ T cells
Azathioprine controls the Rac1-MEK pathway

- **anti-CD3**
  - phospho-MEK: 10% 12%
  - annexin V: 100 101 102 103 104

- **anti-CD3/28**
  - phospho-MEK: 29% 7%
  - annexin V: 100 101 102 103 104

- **anti-CD3/28 + 6-TG**
  - phospho-MEK: 10% 13%
  - annexin V: 100 101 102 103 104

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**Western Blot Analysis**

- **p IκB**: 42 kDa
- **p MEK**: 42 kDa
- **actin**: 42 kDa

**Concentration**

- 0 µmol
- 5 µmol 6-MP

**Other Proteins**

- **IκB**: 42 kDa
- **MEK**: 45 kDa
- **ERK2**: 45 kDa

**Affinity Tag**

- azathio- prime 6-MP
Azathioprine suppresses the vav/Rac1 pathway.

- Rac1-GTP
  - 25 kDa

- anti-CD3  CD3/28

- Vav
  - 95 kDa

- ERK2
  - 42 kDa

- Stat 3

- anti-CD3/28
Azathioprine suppresses the vav/Rac1 pathway
The 6-Thio-GTP hypothesis

Evidence: Detection of 6-TG in Rac1 immunoprecipitates from 6-MP treated T cells
Specificity of 6-ThioGTP binding to Rac1

6-thioguanine-triphosphate (6-Thio-GTP)

radiolabelled GTP

recombinant Rac1

Versus Ras

Measure bound radiolabelled GTP

Thio-GTP (µM)

Relative GTP-binding (% of control)

Rac 1

Ras
Specificity of 6-ThioGTP for Rac1

**Ras**

- CD3
- CD3/28
- CD3/28 + Aza
- CD3/28 + 6-MP
- CD3/28 + 6-TG

25 KD

**Rac1**

- CD3
- CD3/28
- CD3/28 + Aza
- CD3/28 + 6-MP
- CD3/28 + 6-TG

25 KD

**Densitometry Ras**

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<tbody>
<tr>
<td>%</td>
<td>80</td>
<td>100</td>
<td>120</td>
<td>80</td>
<td>100</td>
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**Densitometry Rac1**

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<tr>
<td>%</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>40</td>
<td>60</td>
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</table>
Specificity of 6-ThioGTP for Rac1

**RhoA**

25 KD

|----------|--------|--------|--------------|---------------|---------------|

**Densitometry RhoA**

|----------|--------|--------|--------------|---------------|---------------|

**Cdc42**

25 KD

|----------|--------|--------|--------------|---------------|---------------|

**Densitometry Cdc42**

|----------|--------|--------|--------------|---------------|---------------|
The specificity of azathioprine-induced blockade of Rac1 function as compared to other GTPases (e.g. Ras) is due to the steric interaction between the sulfur atom and the Ala159 residue.
6-ThioGTP blocks vav exchange activity on Rac1

[Graph showing the effect of 6-ThioGTP on vav exchange activity on Rac1.]
Rac1-6ThioGTP-vav model

- Cyan = switch 1 region of Rac1 (residues 27-35)
- Magenta = switch 2 region of Rac1 (residues 59-71)
- Yellow = contact area of vav1 with Rac1
A new model for azathioprine action

azathioprine

6-mercaptopurine (6-MP)

xanthin oxidase

6-thiouric acid

6-thioguanine (6-TG)

TPMT

HPRT

6-methyl-MP

6-thioguanine-triphosphate (6-thio-GTP)

incorporation

Rac1

apoptosis of activated T cells
A model for azathioprine action

CD28

CD28

Vav

GTP

CD28

GDP

CD28

azathioprine

6-MP

6-TG

6-thioGTP

Rac1

thioGDP

Rac1

thioGTP

MEKK1 (MAP3K)

MEK1 (MAPKK)

IKKα

IKKβ

IKKγ

IkBa

NF-κB

p50/p65

antiapoptotic signal

bcl-xL

STAT-3

pSTAT-3

pSTAT-3

STAT-3
Clinical study using 6-ThioGTP levels

Design: prospective study in 50 Crohn’s patients Receiving AZA Therapy

<table>
<thead>
<tr>
<th>TGN</th>
<th>Response</th>
<th>Flares/a</th>
<th>TGDP</th>
<th>TGTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;100</td>
<td>0.42</td>
<td>1.0</td>
<td>29</td>
<td>118</td>
</tr>
<tr>
<td>&lt;100</td>
<td>0.16</td>
<td>2.1</td>
<td>6</td>
<td>54</td>
</tr>
</tbody>
</table>

| >100 Q>90 | 0.71 | 0.21 | 4 | 155 |
| >100 Q<90 | 0.25 | 1.7  | 45 | 93  |
| <100 Q>90 | 0.1  | 1.1  | 4  | 68  |
| <100 Q<90 | 0.3  | 2.6  | 8  | 38  |

TGN - Thioguanine Nucleotides

Teichgräber et al. 2005
Steric Modelling of 6-Thio-GTP Analogues

Status:
12 analogues have been identified
6 analogues have been synthesized
Summary

• Azathioprine specifically blocks vav exchange activity on Rac1 in T cells by incorporation of 6-Thio-GTP

• Azathioprine-induced blockade of Rac1 activation causes suppression of NF-kappaB and STAT3 activation leading to a mitochondrial pathway of apoptosis

• Azathioprine appears to mediate immunosuppression in IBD by inducing T cell apoptosis via 6ThioGTP

• 6ThioGTP levels in erythrocytes correlate with clinical responses to azathioprine

• Selective targeting of apoptosis may be important for designing new therapies for IBD

• Designing of 6-ThioGTP analogues with higher affinity to Rac1 may lead to novel therapies for IBD
Ralf Kiesslich, Martin Holtmann
Jonas Mudter, Raja Atreya
Kai Hildner, Thanka Nadar.
Con Schneider, Jürgen Siebler

Imke Tiede, Christoph Becker
Daniela Poppe, Stefan Wirtz
Benno Weigmann, Alexej Nikolaev
Brigitte Bartsch, Massimo Fantini

Jan Schmidt
Guido Schürmann
Stefan Rose-John
Susanne Strand
Hans A. Lehr
Henning Walczak
Richard Blumberg
Reza Ahmadian
Gerhard Fritz
Xose Bustelo