Molecular mechanisms of B lymphomagenesis induced by TRAF3 inactivation

Ping Xie

Dept. of Cell Biology and Neuroscience, Rutgers University

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The TNFR-associated factor (TRAf) family of adaptor proteins

- TRAF1
- TRAF2
- TRAF3
- TRAF4
- TRAF5
- TRAF6

Zn RING Zn Fingers Coiled-coil (TRAf-N) domain TRAF-C domain
Shared use of TRAF3 by the TNF-R superfamily

EBV-encoded oncoprotein LMP1

CD40

TRAF3

BAFF-R

TACI

BCMA

4-1BB

CD27

RANK

OX40

GITR

LTβR

HEM

Troy

XEDAR

TNFR2

TNFR1

EDAR

FAS

TRAIRR1

TRAIRR2

DR3

DR6

NGFR

Plasma membrane

Cysteine-rich domain

Death domain

TRAF binding motif
Mice genetically deficient in TRAF3 show early lethality


- die by 10 days of age.
- have smaller lymphoid organs, and exhibit a progressive depletion in all lineages of white blood cells in the periphery.

We generated conditional TRAF3 knockout mice (TRAF3\(^{\text{flox/flox}}\)) as a model system to investigate the *in vivo* function of TRAF3.

TRAF3\(^{\text{flox/flox}}\)CD19\(^{+/\text{Cre}}\): B cell-specific TRAF3\(^{-/-}\) (B-TRAF3\(^{-/-}\)) mice
B-TRAF3-/- mice exhibit enlarged spleen and lymph nodes

- Prolonged survival of mature B cells
- Constitutive NF-κB2 activation

Autoimmune manifestations

TRAF3 mutations in human patients with B cell malignancies

- Homozygous deletions and inactivating mutations of the TRAF3 gene
  - multiple myeloma (MM)
  - splenic marginal zone lymphoma (SMZL)
  - B cell chronic lymphocytic leukemia (B-CLL)
  - mantle cell lymphoma (MCL)
  - Waldenström’s macroglobulinemia (WM)

TRAF3: a tumor suppressor gene in B cells?

Hypothesis: B-TRAF3<sup>−/−</sup> mice may spontaneously develop B lymphoma as they age.

Carissa Moore
**B-TRAF3−/− mice spontaneously develop B lymphomas**

<table>
<thead>
<tr>
<th>Mice examined</th>
<th>Number of mice with B lymphomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>(total number)</td>
<td>Spleen  Ascites  BM  CLNs  MLNs  Kidney  Lung  Liver</td>
</tr>
<tr>
<td>Mice without overt external symptoms</td>
<td>20  6  7  5  5  4  3  0</td>
</tr>
<tr>
<td>n=32</td>
<td></td>
</tr>
<tr>
<td>Moribund mice</td>
<td>18  9  9  3  0  5*  6  3</td>
</tr>
<tr>
<td>n=18</td>
<td></td>
</tr>
</tbody>
</table>

Moore et al., *Leukemia*, 26: 1122-1127, 2012
TRAF3<sup>−/−</sup> B lymphomas were distinguished from normal B lymphocytes

Flow cytometric analysis of splenic B cells

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Southern blot of IgH

Moore et al., *Leukemia*, 26: 1122-1127, 2012
### TRAF3⁻/⁻ B lymphomas do not contain somatic hypermutation in the IgH gene V(D)J region

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Organ</th>
<th>IgH V gene</th>
<th>Frequency</th>
<th>Somatic hypermutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>7041-10</td>
<td>Spleen</td>
<td>VH36-60.a2.90</td>
<td>18/20</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VH7183.a19.31</td>
<td>2/20</td>
<td>No</td>
</tr>
<tr>
<td>7060-8</td>
<td>Spleen</td>
<td>VH7183.a25.43 (or VH283)</td>
<td>8/19</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VH36-60.a2.90</td>
<td>11/19</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Ascites</td>
<td>VH7183.a25.43 (or VH283)</td>
<td>11/18</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VH36-60.a2.90</td>
<td>6/18</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VH7183.a47.76</td>
<td>1/18</td>
<td>No</td>
</tr>
<tr>
<td>5-5</td>
<td>Spleen</td>
<td>VH98-3G (VH7183.a21.35)</td>
<td>15/21</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VS107.a3.106</td>
<td>3/21</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VH7183.a2.3</td>
<td>1/21</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VH36-60.a2.90</td>
<td>1/21</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VH7183.a7.10</td>
<td>1/21</td>
<td>No</td>
</tr>
<tr>
<td>7079-8</td>
<td>Ascites</td>
<td>VH7183.a2.3 (7183.2.3)</td>
<td>18/21</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V98-3G</td>
<td>2/21</td>
<td>K16?</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VH7183.a28.48</td>
<td>1/21</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**B-TRAF3⁻/⁻** mice spontaneously develop marginal zone lymphoma (MZL) or B1 lymphomas.
TRAF3⁻/⁻ B lymphoma cells are transplantable in immunodeficient NOD SCID recipient mice

- Immortalized cell lines
  » 105-8
  » 27-9
  » 115-6
Identification of secondary oncogenic hits involved in TRAF3 inactivation-induced B lymphagenesis

Microarray analyses: Dr. Ronald Hart

TRAF3 deletion ➔ NF-κB2 activation ➔ Premalignant TRAF3-/- B cell ➔ 2º hits ➔ B lymphoma

- 160 up-regulated genes
- 244 down-regulated genes

Up-regulation verified by Taqman qPCR:

MCC, Sox5, Diras2, Tbc1d9, Ccbp2, Btbd14a, Sema7a, Twsg1, Ppap2b, TCF4, Tnfrsf19, Zcwpw1, and Abca3, etc.
Sox5 and MCC are aberrantly up-regulated in TRAF3-/- mouse B lymphomas

A novel isoform of Sox5


MCC: Mutated in colorectal cancer
Evidence led us to further study MCC

- MCC was identified as a tumor suppressive gene in colorectal cancer. However, the function of MCC in B cells has not been studied.

- MCC is aberrantly up-regulated in TRAF3-deficient mouse B lymphomas and human patient-derived MM cell lines.

- Aberrant MCC up-regulation is frequently detected in a variety of primary human B cell neoplasms.
  - PEL, CBL, DLBCL, BL, and MM

- MCC expression was not detected in normal or premalignant TRAF3−/− B cells even after treatment with B cell stimuli.

- Lentiviral shRNA vector-mediated knockdown of MCC induced apoptosis and inhibited proliferation in human MM cells.
MCC is mainly localized at mitochondria in human MM cells

- The ER stress inducers DTT and thapsigargin induced apoptosis and decreased MCC protein levels in human MM cells.

ER stress inducers
- DTT
- Thapsigargin (Thg)
MCC regulates different signaling pathways in human MM cells versus other cancers

- Known MCC targets in colorectal cancer and hepatocellular carcinoma
  - Phospho-β-catenin, β-catenin, IκBα, IκBβ, and RelA

- Additional regulators not changed by MCC knockdown or overexpression:
  - Bcl2, Bim, Bad, Bid, Bik
  - cyclin D1, cyclin D2, p21, E2F1, p53
  - P-p38, P-JNK, P-Akt

- MCC downstream targets in human MM cells
  - Mcl-1, caspase 8, and caspase 3
  - p27, cyclin B1, Phospho-ERK, c-Myc
MCC interacting proteins in human MM cells

• Lentiviral expression vectors of MCC for immunoprecipitation
  – pUB-FLAG-hMCC
  – pUB-hMCC-SBP-6xHis

• Known MCC interacting partners in colorectal cancer and 293T cells
  – β-catenin, Mst3, VCP, PP2A, DFFA, VHL, VDAC, scribble, myosin IIb, etc.

LC-MS/MS: Dr. David Perlman, Princeton University

➢ 365 proteins of the MCC-interactome

Previously known MCC interactors: 255
MCC interactors in whole lysates: 333
MCC interactors in mitochondria: 207
Functional clustering of the MCC-interactome
Identified from human MM cells

- MCC interactors in whole lysates: 333
  - Cell Death and Survival (123)
  - Cellular Growth and Proliferation (120)
  - DNA Replication and Repair (39)
  - Molecular Transport (34)
  - Protein Trafficking (17)

- Disease association analysis by Ingenuity: cancer
  - 195 of the 333 (58.5%) MCC interactors in whole lysates
  - 91 of the 207 (43.9%) MCC interactors in mitochondria
PHB2, a mitochondrial protein critical for survival, was identified as an MCC-interacting protein in human MM cells.

PHB2_Human: 64.21% coverage
PARP1 and PHB2 were co-immunoprecipitated with MCC in human MM cells

- **PARP1**: the top novel MCC-interacting protein
- **PHB2**: the top previously known MCC-interacting protein
- Both are known regulators of cell survival and proliferation.
- Both regulate the MCC targets identified by knockdown and overexpression
  - Phospho-ERK, cyclin B1, p27, c-Myc
  - Mcl-1, caspase 8, and caspase 3

Complex mechanisms of TRAF3 inactivation-mediated oncogenic survival and malignant transformation of B cells

TRAF3 deletion → NF-κB2 activation → Normal B cell → Premalignant TRAF3-/- B cell → 2° hits (MCC, Sox5) → B lymphoma

<table>
<thead>
<tr>
<th>Genetic Data analysis</th>
<th>RNA-Seq</th>
</tr>
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<tbody>
<tr>
<td>Epigenetic Global changes</td>
<td>ChIP-Seq</td>
</tr>
<tr>
<td>Epigenetic Global changes</td>
<td>ChIP-Seq</td>
</tr>
<tr>
<td>Expression mRNA</td>
<td>160 ↑ and 244 ↓ genes</td>
</tr>
<tr>
<td>microarray</td>
<td>microarray</td>
</tr>
<tr>
<td>Metabolic Data analysis</td>
<td>LC-MS</td>
</tr>
</tbody>
</table>
Translational study

- Diagnostic markers: TRAF3 inactivation, Rhbdf1, Sox5, MCC, etc.
- Therapeutic targets: NF-κB2, PKCδ, Rhbdf1, PC, MCC, etc.

To test: PARP inhibitors, PHB2 ligands, etc.

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