ANNUAL REPORT

STUDENT: **LAVINIA MARTUSCELLI** YEAR: **2014-2015**

DIVISION OF HEMATOLOGY CYCLE**: XXIX**

TUTOR: **DOTT. DAVIDE ROSSI**

NON CANONONICAL NF-κB SIGNALING IN B-CELL LYMPHOPROLIFERATIVE DISORDERS

***Introduction*.** The non-canonical NF-κB pathway, involved in physiology of mature B-cell lymphocytes, is engaged by a group of tumor necrosis factor (TNF) receptors, such as CD40 and BAFFR [1-2]. Upon receptor binding, the TRAF3/MAP3K14-TRAF2/BIRC3 negative regulatory complex of non-canonical NF-κB signaling is disrupted, allowing the cytoplasmic release and stabilization of MAP3K14 (also known as NIK), the central activating kinase of non-canonical NF-κB signaling [3]. The stabilized MAP3K14 activates the IKKα kinase, which in turns directly phosphorylates NF-κB_2 /p100, inducing partial proteolysis of p100 to p52 by the proteasome. The p52 protein dimerizes with RelB to translocate into the nucleus, where it regulates gene transcription [4]. The *BIRC3* gene, a master negative regulator of non-canonical NF-κB signaling, is recurrently mutated in ~5% chronic lymphocytic leukemia (CLL) and ~10% splenic marginal zone lymphoma (SMZL) [5-6]. *BIRC3* inactivating mutations (frameshift or non-sense substitutions) cause the truncation of the C-terminal RING domain of the BIRC3 protein, whose E3 ubiquitin ligase activity is essential for proteasomal degradation of MAP3K14 [6]. This project aims at characterizing the functional consequences of *BIRC3* mutations on the biochemistry of non-canonical NF-κB signaling and on the cellular phenotype in CLL and SMZL.
For the functional experiments, we used different cell line models of CLL (MEC1) and SMZL (KARPAS 17-18, SSK41, VL51). The MAVER-1 and Z-138 cell lines of MCL, which harbor genetic activation of non-canonical NF-kB signaling, were utilized as positive controls, while the JEKO-1 cell line of MCL and the human embryonic kidney (HEK) 293T cell line, as negative controls [7].

***Genetic characterization of BIRC3, TRAF2 and TRAF3*.** The key regulators genes of non-canonical NF-κB pathway (*BIRC3*, *TRAF2*, *TRAF3*) were screened for mutations and gene deletions in our panel of cell lines by Sanger sequencing and FISH (Fluorescence *In Situ* Hybridization), respectively. The MEC1 and VL51 cell lines carried heterozygous mutations that

truncate the C-terminal RING domain of BIRC3 protein, the KARPAS 17-18 cell line harbored a monoallelic deletion of the entire *BIRC3* locus, while the SSK41 cell line was wild type.

Biochemical dissection of the non-canonical NF-κB pathway and gene expression signature. In the VL51 and MEC1 cell lines, harboring a mutated *BIRC3*, non-canonical NF-κB signaling was active, as documented by the stabilization of MAP3K14, phosphorylation of NF-κB_2 and its processing from p100 to p52. Conversely, the *BIRC3* wild type SSK41 and KARPAS 17-18 cell lines were devoid of biochemical clues of non-canonical NF-κB signaling. Consistent with the molecular clues of non-canonical NF-κB activation, the gene expression signature of the VL51 and MEC1 cell lines was significantly enriched of NF-κB target genes.

Western blot of cytoplasmatic and nuclear fractions of p52. To complete the biochemical dissection of the non-canonical NF-κB signaling in our panel of SMZL and CLL cell lines, we assessed the localization of p52 by Western blotting after fractionation of cytoplasmic and nuclear proteins (Qproteome Nuclear Protein Kit, Qiagen). Western blot analysis, using antibody directed against p52 protein (Cell Signaling, #4882), revealed a nuclear localization of p52 in the VL51 and MEC1 cell lines, which harbored *BIRC3* mutations. MAVER-1 and Z-138 cell lines were used as positive controls, while JEKO-1 and HEK293T cell lines were utilized as negative controls [7]. Beta-tubulina (Sigma Aldrich, #T5201) and BRG1 (Santa Cruz Biotechnology, #17796) were used as controls for the purity of nuclear and cytoplasmic fractions, respectively.

Treatment with the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib. Consistent with the ability of *BIRC3* mutations to activate NF-κB independent of BTK [7], a tyrosine kinase at the crossroad of B-cell receptor (BCR) and NF-kB signaling [8], cell lines harboring *BIRC3* mutations were resistant to the BTK inhibitor ibrutinib.

Western blot studies of primary CLL and SMZL samples. To validate in vivo in patients the results our observations from cell lines, we identified 3 primary CLL and SMZL samples known to harbor heterozygous inactivating mutations of *BIRC3*. Case 9321 (CLL) carried a frameshift mutation of *BIRC3* exon 9 (c.1638_1639insA p.Q547fs*12). Case 14462 (CLL) carried frameshift mutations of *BIRC3* exon 6 (c.1282delA p.R428fs*19) and exon 8 (c.1609delG p.E537fs*3). Case 12603 (SMZL) carried a nonsense truncating mutation (c.1639C>T p.Q547*) and 2 deletions: one that removed 5 bp of *BIRC3* exon 6 (c.1283_1287delGGGAA p.R428fs*8) and another that removed 6 bp of *BIRC3* exon 9 (c.1786_1791delAAGGGT p.K596del2aa). Only the first deletion causes frameshift in the gene-coding sequence. *BIRC3* mutations of cases 9321, 14462 and 12603 were predicted to generate aberrant transcripts carrying premature stop codons and lacking the C-terminal RING domain, whose E3 ubiquitin ligase activity is essential for proteasomal degradation of MAP3K14 [3-9-10]. Two *BIRC3* wild type cases, namely 11731 (SMZL) and 12600 (SMZL), were used as controls.

By Western blotting of proteins from primary tumor cells, case 9321, which harbored a monoallelic mutation truncating *BIRC3*, expressed both wild type BIRC3 protein as well as an additional aberrant band of lower molecular weight corresponding in size to the predicted mutant BIRC3. Case 14462, which harbored two truncating mutations in two different alleles of *BIRC3*, expressed two

aberrant bands of molecular weight corresponding in size to the predicted truncated BIRC3, while was devoid of the wild type BIRC3. Case 12603 had a mosaic subclonal architecture of the *BIRC3* gene, because harbored 3 BIRC3 truncating mutations resulting in the expression of 3 aberrant bands of lower molecular weight corresponding in size to the predicted mutant BIRC3 along with the wild type protein. *BIRC3* mutated cases (9321, 14462 and 12603) showed non-canonical NF-κB signaling, as documented by the stabilization of MAP3K14 and NF-κB_2 processing from p100 to p52. In contrast, cases 11731 and 12600, which were devoid of *BIRC3* mutations, lacked of MAP3K14 stabilization and NF-κB_2 processing from p100 to p52, consistent with the absence of non-canonical NF-κB signaling.

Immunohistochemistry of primary SMZL biopsy samples. To extend the validation in vivo in patients of the results our observations from cell lines, we collected 38 paraffin-embedded primary SMZL spleen tumor tissues and screened them for mutations of *BIRC3* (exons 2-9, including splicing sites; RefSeq NM_001165.3) and *TRAF3* (exons 2-11, including splicing sites; RefSeq NM_145725.2) by Sanger sequencing. Overall, *BIRC3* was affected by truncating mutations in 2/38 (5%), while *TRAF3* a second negative regulator of non-canonical NF-κB, was disrupted by mutations in 2/38 (5%) SMZLs. By immunohistochemical assay using anti-NF-κB2 (p52; Cell Signaling, #3017) antibody, all cases with *BIRC3* or *TRAF3* mutations (4/4, 100%) showed a clear nuclear staining for NF-κB2 (p52), consistent with an activation of non-canonical NF-κB. Nuclear localization of NF-κB2 (p52) was also detected in 8/34 (23%) cases that were wild type for *BIRC3* and *TRAF3*, suggesting that additional and yet unknown molecular mechanisms might activate non-canonical NF-κB in these cases.

Knockdown of MAP3K14 by RNA interference. To test whether *BIRC3* mutated cells are addicted of MAP3K14 (NIK) overexpression, we produced lentiviruses expressing 3 short hairpin RNAs (shRNAs) targeting MAP3K14, as well as the scrambled shRNA, cloned into the BamHI/HindIII cloning sites of the pGFP-C-shLenti vectors (OriGene Technologies). Within the 5'-LTR and 3'-LTR regions, each pGFP-C-shLenti vector contains an shRNA expression cassette driven by an U6 promoter, a puromycin resistance marker driven by a SV40 promoter and a GFP driven by a CMV promoter. The shRNA expression cassette consists of 29 bp target-gene-specific sequence, a 7 bp loop, and another 29 bp reverse complementary sequence, followed by a TTTTTT termination sequence. The HEK293T cell line was co-transfected with expression (3 different pGFP-C-MAP3K14-shLenti or pGFP-C-non-effective-shLenti) vectors and adjuvant vectors (pMDL, REV and VSV-G). Fluorescence microscope was utilized to check the expression of the GFP in the transfected HEK293T cell line. After virus titration, the VL51 cell line was infected with lentiviruses harboring the shRNAs against MAP3K14 and the scrambled through a spinoculation protocol. After four days, infected cells were monitored by flow cytometry for the expression of the GFP and were selected by puromycin (1.5 μg/mL). Through this approach, VL51 cells stably expressing the shRNAs against MAP3K14 or the scrambled constructs were established. This set up experiment allowed to identify the shRNA vector with the highest efficacy in silencing MAP3K14, both by Western blotting and flow cytometry.

***Perspectives*.** The consequences of MAP3K14 knockdown by RNA interference will be assessed at the biochemical level on non-canonical NF-κB signaling, at the gene expression level on the non-canonical NF-κB-associated gene signature, and at the cellular level on cell survival.

***Conclusions*.** These data indicate that:

1. *BIRC3* mutations lead to constitutive non-canonical NF-κB signaling activation in the *BIRC3* mutated VL51 (SMZL) and MEC1 (CLL) cell lines as documented by the stabilization of MAP3K14, phosphorylation of NF-κ$B_{2,}$ its processing from p100 to p52, nuclear localization of p52 and upregulation of NF-κB target genes;
2. cell lines harboring *BIRC3* mutations are resistant to ibrutinib, consistent with the ability of *BIRC3* mutations to activate NF-κB independent of BTK;
3. Western blotting for MAP3K14 expression and immunohistochemistry for p52 nuclear localization confirms non-canonical NF-κB signaling activation also in *BIRC3* mutated primary SMZL and CLL tumors.

References

[1] Coope HJ, et al. CD40 regulates the processing of NF-κB2 p100 to p52. EMBO J 2002; 21:5375–5385.

[2] Claudio E, Brown K, Park S, Wang H, Siebenlist U. BAFF-induced NEMO-independent processing of NF-κB_2 in maturing B cells. Nat Immunol 2002; 3:958–965.

[3] Vallabhapurapu S, Karin M. Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol 2009; 27:693–733.

[4] Staudt LM. Oncogenic activation of NF-kappaB. Cold Spring Harb Perspect Biol 2010; 2:a000109.

[5] Rossi D, Fangazio M, Rasi S, Vaisitti T, Monti S, Cresta S, et al. Disruption of *BIRC3* associates with fludarabine chemorefractoriness in TP53 wild-type chronic lymphocytic leukemia. Blood 2012; 119(12):2854–62.

[6] Rossi D, Deaglio S, Dominguez-Sola D, Rasi S, Vaisitti T, Agostinelli C, Spina V, Bruscaggin A, Monti S, Cerri M, et al. Alteration of *BIRC3* and multiple other NF-κB pathway genes in splenic marginal zone lymphoma. Blood 2011; 118:4930–4934.

[7] Rahal R, et al. Pharmacological and genomic profiling identifies NF-κB–targeted treatment strategies for mantle cell lymphoma. Nat Med 2014; 20(1):87–92.

[8] Woyach JA, Johnson AJ, Byrd JC. The B-cell receptor signaling pathway as a therapeutic target in CLL. Blood 2012; 120:1175-84.

[9] Conze DB, Zhao Y, Ashwell JD. Non-canonical NF-kappaB activation and abnormal B cell accumulation in mice expressing ubiquitin protein ligase-inactive c-IAP2. PLoS Biol 2010; 8(10):e1000518.

[10] Zarnegar BJ, Wang Y, Mahoney DJ, et al. Non canonical NF-kappaB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. Nat Immunol 2008; 9(12):1371-1378.

Attended seminars

1. 03/09/2015 Prof. Darko Boshnakovski, PhD - University Goce Delcev Stip, Faculty of Medical Sciences (Stip, R. Macedonia). “Cell based models for studying molecular mechanisms of Facioscapulohumeral Muscolar Distrophy (FSHD)”, “Toward animal model for Facioscapulohumeral Muscolar Distrophy (FSHD)”
2. 28/07/2015 Dr. Vincenzo Cantaluppi, MD - Facoltà di Medicina e Chirurgia, Università di Torino (Italy). “Le cellule staminali nel danno renale acuto e nel trapianto di rene”
3. 09/07/2015 Dr Gwenaël ROLIN, PhD - Clinical Research Engineer - Thomas LIHOREAU - Ingénieur hospitalier, Research and Studies Center on the Integument (CERT), Department of Dermatology, Clinical Investigation Center (CIC INSERM 1431), Besançon University Hospital; INSERM UMR1098, FED4234 IBCT, University of FrancheComté, Besançon, France. Miniworkshop on “Biotechnology for Dermatology”
4. 10/06/2015 Prof. Nicoletta Filigheddu - Università del Piemonte Orientale (Italy) “Basis of scientific research”
5. 05/06/2015 Mariet C.W. Feltkamp - Associate Professor of Medical Virology, Department of Medical Microbiology, Leiden University Medical Center (Leiden, The Netherlands). “Recent developments in (cutaneous) Human Polyomavirus research”
6. 25/05/2015 Prof. Steve Ellis - Medical School, University of Louisville (Kentucky) “Ribosomopathies”
7. 07/05/2015 Prof. John McDonald, MD, PhD - Integrated Cancer Research Center, School of Biology and Parker H. Petit Institute of Bioengineering and Bioscience, Georgia Institute of Technology, Georgia Tech University, Georgia (Atlanta, USA). “An integrated approach to the diagnosis and treatment of ovarian cancer”
8. 11/03/2015 Prof. Darko Bosnakovski - Associate Professor, University "Goce Delcev" Stip, Faculty of Medical Sciences, Krste Misirkov bb, 2000 Stip R. Macedonia. “Proof of principle for cell therapy: from autologous transplantation of tissue specific progenitors to gene corrected patient specific injured pluripotent stem cells”
9. 27/01/2015 Prof. Antonio Sica - DiSCAFF, UPO, Novara. “Myeloid cells as therapeutic target in cancer”
10. 20/01/2015 Dott. Tonino Alonzi, PhD, Lab. Of Gene Expression and Experimental Hepatology, Istituto Nazionale per le Malattie Infettive “L. Spallanzani” IRCCS, Rome. “Different molecular mechanisms regulate hepatocyte differentiation during the transitions between epithelial and mesenchymal states”
11. 19/01/2015 Prof. Dr Yong-Sang Song, MD, PhD Director Cancer Research Institute, Gynecologic Oncology Chariman, Cancer Biology Interdisciplinary Program Professor, Obstetrics and Gynecology, College of Medicine Seoul National University. “Anticancer strategy Targeting cancer cell metabolism in ovarian cancer”
12. 17/12/2014 Dott. Roberto Furlan, San Raffaele University, Milan. “Microglia microvescicles: messengers from the diseased brain”
13. 28/11/2014 Dott. Arvind Patel - Programme Leader, MRC Centre for Virus Research, University of Glasgow (UK). “Humoral responses to HCV infection and clinical outcomes”

14. 27/11/2014 Prof. Laura Baglietto - Inserm - Centre for Research in Epidemiology and Population Health, Unit: Nutrition, Hormones and Women's Health, Paris. "Nuove sfide ed opportunità dell'epidemiologia molecolare per lo studio dei tumori"
15. 14/11/2014 Dott.ssa Francesca Boccafoschi - Department of Health Sciences, University of Eastern Piedmont. "Tissue engineering: the state of the art"
16. 06/11/2014 Prof. Andrew L. Snow - Department of Pharmacology Uniformed Services University of the Health Sciences Bethesda (Maryland, USA). "Dysregulated antigen receptor signaling: molecular lessons from two congenital lymphoproliferative disorders"

Partecipations to conferences

45° Congresso Nazionale della Società Italiana di Ematologia (SIE). 4-7 Ottobre 2015, Firenze. "*BIRC3* mutated B-cell lymphoproliferative disorders are addicted of non canononical NF-κB signaling"

Publications

Spina V, Martuscelli L, Rossi D. Molecular deregulation of signaling in lymphoid tumors. Eur J Haematol. 2015; 95(4): 257-69.