ANNUAL REPORT

STUDENT: **LAVINIA MARTUSCELLI** YEAR: **2013-2014**

DIVISION OF HEMATOLOGY CYCLE**: XXIX**

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

***Introduction*.** The human genome era heralded a fundamental progress in the field of lymphoma genetics that shifted from a candidate gene approach toward global views of genomes and transcriptomes. This shift was made possible by increasingly powerful experimental and analytic methodologies, and has led to a burst of disease-relevant molecular information in a variety of lymphoid tumors [1], including chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), and splenic marginal zone lymphoma (SMZL). From a pathogenetic standpoint, these progresses have contributed to understanding the genetic bases and complexity of lymphomagenesis [2], and have provided insights into pathways not previously known to be involved in these tumors. From a clinical standpoint, these studies have created new paradigms to address currently unmet clinical needs in the management of lymphoma, by providing novel biomarkers for the refinement of diagnosis and stratification, and novel molecular targets for therapeutic approaches. As global views of lymphoma genomes have emerged, the magnitude of their complexity has become expressly manifest. Lymphoma coding genomes may harbor dozens of mutations. However, only a subset of this large number of alterations is currently known to contribute to cancer phenotypes. Thus, intense efforts in recent years have focused on the identification of driver mutations that decisively influence the viability and clinical behavior of a given lymphoma type.

In CLL, SMZL and MCL, malignant B-cells manipulate signaling pathways that are central to normal B-cell homeostasis. Among signaling pathways that are central for the physiology of mature B-cell lymphocytes, non-canonical NF-kB (nuclear factor κB) signaling plays a key role in a wide range of B-cell functions, such as cell survival, proliferation, differentiation and promotion of both innate and acquired immunity [3-4].

The non-canonical NF-κB pathway is engaged by a select group of tumor necrosis factor (TNF) receptors, such as CD40R and BAFFR [5-6]. 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, the central activating kinase of non-canonical NF-κB signaling [7]. 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 [8]. The *BIRC3* gene, a master negative regulator of non-canonical NF-κB signaling, is recurrently mutated in ~5% CLL, ~10% SMZL and ~10% MCL [9-10-11]. *BIRC3* inactivating mutations are mainly represented by frameshift or non-sense substitutions causing the truncation of the C-terminal RING domain of the BIRC3 protein, whose E3 ubiquitin ligase activity is required to prime MAP3K14 towards proteasomal degradation [10]. Beside *BIRC3*, also *TRAF2* and *TRAF3*, other components of the TRAF3/MAP3K14-TRAF2/BIRC3 negative regulatory complex of non-canonical signaling, are targeted in CLL, SMZL and MCL. The predicted functional consequence of *BIRC3* mutations in B-cell tumors is MAP3K14 stabilization in the cytoplasm and the constitutive activation of non-canonical NF-κB signaling [10].
In B-cells, the Bruton's tyrosine kinase (BTK) is at the crossroad of B-cell receptor (BCR) and NF-kB signaling [12]. Upon BCR activation by the microenvironment, BTK transduces the signal to the canonical NF-κB pathways, resulting in prevention of apoptosis, as well as in promotion of cell adhesion, cell migration, and other cellular processes that can help survival and proliferation of tumor cells. This strong biological rationale makes BTK an ideal target for therapy of B-cell malignancies. Ibrutinib is a selective tyrosine kinase inhibitor that covalently and irreversibly binds BTK and, consequently, blocks survival, proliferation, and migration of B-cell tumor cells in *in vitro* models of the tumor microenvironment [13]. Recently, ibrutinib has been shown to exert impressive clinical activity in CLL and MCL [14-11]. In the context of MCL, molecular alterations activating the non-canonical NF-κB signaling have been associated with resistance to ibrutinib [11].
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.

Identification of CLL and SMZL cell lines models. To determine the functional relevance of *BIRC3* mutations in CLL and SMZL, we used different cell line models of CLL (MEC1) and SMZL (KARPAS 17-18, SSK41, VL51). For the functional experiments, 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 were utilized as negative controls [11].

Molecular studies. To investigate the molecular basis non-canonical NF-κB signaling of our cell line models, key regulators genes of non-canonical NF-κB pathway were screened for mutations and CNAs. Mutation analysis of non-canonical NF-κB pathway key regulators, including *BIRC3* (exons 2-9, including splicing sites; RefSeq NM_001165.3), *TRAF2* (exons 2-11, including splicing sites; RefSeq NM_021138.3), *TRAF3* (exons 3-12, including splicing sites; RefSeq NM_145725.2), was performed by DNA Sanger sequencing. Copy number abnormalities (CNAs) of selected candidate genes were assessed by FISH (Fluorescence *In Situ* Hybridization) analysis, using the following probes: RP11-177O8 (*BIRC3*), RP11-769N4 (*TRAF2*), RP11-676M2 (*TRAF3*).
The *BIRC3* gene was targeted by heterozygous inactivating mutations in the MEC1 and VL51 cell lines. The MEC1 cell line carried a large deletion that removed 537 bp of *BIRC3* exon 2 (c.267_803del537bp p.L89del179aa), causing frameshift in the gene-coding sequence. The VL51 cell line carried a deletion that removed 14 bp of *BIRC3* exon 6 (c.1299_1302del4bp p.E433fs*13),

causing a frameshift in the gene-coding sequence. *BIRC3* mutations of the MEC1 and VL51 cell lines 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 [7-15-16]. The KARPAS 17-18, MAVER-1 and Z-138 cell lines carried monoallelic deletions of the entire *BIRC3* locus that were clonally represented in all cases (100% of the cells). The Z-138 cell line harbored a nonsense mutation (c.341G>A p.W114*) in the *TRAF2* gene, which was predicted to eliminate the C-terminal portion of the protein and the docking site for BIRC3 [16]. The MAVER-1 cell line harbored a biallelic *TRAF3* deletion in 100% of the cells. TRAF3 binds MAP3K14 and is required for MAP3K14 recruitment to BIRC3 degradation [7-16-17-18].

Western blot studies. The entire non-canonical NF-κB pathway was assessed at the biochemical level in the KARPAS 17-18, SSK41, VL51 and MEC1 cell lines using the following antibodies: BIRC3 (Cell Signaling, #3130), TRAF2 (Cell Signaling, #4712), TRAF3 (Cell Signaling, #4729), MAP3K14 (Cell Signaling, #4994), Phospho-NF-κB$_2$ p100 (Cell Signaling, #4810), NF-κB2 p100/p52 (Cell Signaling, #4882). Alpha-actina (Santa Cruz Biotechnology, #sc-1615) was used as loading control. MAVER-1 and Z-138 cells were used as positive controls, because they harbor a genetically activated non-canonical NF-κB signaling, while JEKO-1 and HEK293T cells were utilized as negative controls [11].

Western blot analysis, using antibody directed against the N-terminus of the BIRC3 protein, revealed the expression of the wild type *BIRC3* allele in the KARPAS 17-18, SSK41,VL51, MEC1, MAVER-1 and JEKO-1 cell line models, which was consistent with the retention of at least one wild type allele in these cells. The VL51 and MEC1 cell lines, which harbored a mutation in the second allele of *BIRC3*, expressed one additional aberrant band of lower molecular weight corresponding in size to the predicted truncated BIRC3. The Z-138 cell line, which harbored a mutation that disrupted *TRAF2*, did not express neither TRAF2 nor BIRC3, consistent with the function of TRAF2 as docking site for BIRC3. The MAVER-1 cell line did not express TRAF3 because of the biallelic deletion of its locus.

In the VL51 and MEC1 cell lines, 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 revealed in these cells. These molecular clues of non-canonical NF-κB signaling activation were consistent with those observed in the Z-138 and MAVER-1 cells, which are known to be addicted of this pathway. In contrast, non-canonical NF-κB activation was not observed in the KARPAS 17-18 and SSK41 cell lines, which were devoid of *BIRC3* mutations, as documented by the lack of MAP3K14 stabilization, phosphorylation of NF-κB$_2$ and its processing from p100 to p52 in these cells. 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.

In order to complete the biochemical dissection of the non-canonical NF-κB nuclear localization of p52 and RelB, will be assessed in our cell line models by Western blotting after fractionation of cytoplasmic and nuclear proteins.

Results achieved in cell lines will be validated in primary CLL and SMZL samples harboring *BIRC3* mutations.

Treatment with ibrutinib. Molecular alterations activating the non-canonical NF-κB signaling have been associated with resistance to ibrutinib. To define the impact of non-canonical NF-κB pathway lesions on ibrutinib senistivity in CLL and SMZL, the SSK41, KARPAS 17-18, VL51 and MEC1 cell lines were treated with 1 μM, 5 μM and 10 μM of ibrutinib or vehicle (DMSO). The JEKO-1 cell line, which is known to be sensitive to ibrutinib [11], was included in the experiment as positive control, while the MAVER-1 and the Z-138 cell lines, which are known to be resistant to ibrutinib [11], were used as negative controls. Relative growth was determined by a Cell-Titer Glo (CTG) Luminescent Cell Viability Assay, 72 h and 96 h after treatment. By this experiment, the VL51 and MEC1 cell lines, which harbored *BIRC3* mutations and activated non-canonical NF-κB signaling, were resistant to ibrutinib, while sensitivity to this drug was maintained in the *BIRC3* wild type KARPAS 17-18 and SSK41 cell lines.

Lentiviral production. In order to overexpress a wild type *BIRC3* gene in the *BIRC3* mutant VL51 and MEC1 cell lines, lentiviral vectors were produced in collaboration and under the supervision of Prof. Antonia Follenzi. The most recurrent *BIRC3* (c.1639delC p.Q547fs*21) mutation was generated into a FLAG-tagged *BIRC3* using the QuickChange® XL Site-Directed Mutagenesis kit (Stratagene). Site-directed mutagenesis was confirmed by DNA Sanger sequencing. FLAG-tagged wild type and mutant *BIRC3* were cloned into the SalI/SmaI sites of a bi-directional pLenti PGK-CMV-Puro-Ires-GFP vector. The correct insertion of the wild-type and mutated FLAG-*BIRC3* within the backbone vector was verified both by enzymatic digestion and DNA Sanger sequencing. The HEK293T cell line, that does not express *BIRC3* at the basal level, was transfected with the pLenti PGK-CMV-Puro-Ires-GFP (empty), pLenti PGK-FLAG-wild-type *BIRC3*-CMV-Puro-Ires-GFP and pLenti PGK-FLAG-mutant *BIRC3*-CMV-Puro-Ires-GFP vectors. Confocal microscopy and treatment with puromycin were utilized to assess the expression of the GFP and the resistance to puromycin, respectively, in the transfected HEK293T cells. Western blot analysis, using antibody directed against the N-terminus of the FLAG-BIRC3 protein, confirmed that both the wild type and mutant FLAG-*BIRC3* proteins were expressed by the vectors in the transected HEK293T cells. Lentiviral stocks were produced by co-transfecting expression (pLenti PGK-CMV-Puro-Ires-GFP, pLenti PGK-FLAG-wild-type *BIRC3*-CMV-Puro-Ires-GFP or PGK-FLAG-mutant *BIRC3*-CMV-Puro-Ires-GFP) vectors and adjuvant vectors (pMDL, REV and VSV-G) into HEK293T cells. After virus titration, the VL51 cell line was infected with vectors harboring the wild-type FLAG-*BIRC3*, the mutant FLAG-*BIRC3* and the empty vector, through a spinoculation protocol. Forty-eight hours after infection, infected cells were selected by puromycin and were monitored by flow cytometry for the expression of the GFP. Through this approach, VL51 cells stably expressing the wild-type FLAG-*BIRC3*, the mutant FLAG-*BIRC3* or the empty vectors were established. Such cell line models will be used to establish the effects of the reconstitution of the wild type *BIRC3* on non-canonical NF-κB signaling and cell viability.

References

[1] Braggio E, Egan JB, Fonseca R, Stewart AK. Lessons from next-generation sequencing analysis in hematological malignancies. Blood Cancer J 2013; 3:e127.

[2] Rampal R, Levine RL. Leveraging cancer genome information in hematologic malignancies. J Clin Oncol 2013; 31(15):1885–1892.

[3] Gerondakis S, Banerjee A, Grigoriadis G, Vasanthakumar A, Gugasyan R, Sidwell T, Grumont RJ. NF-kappaB subunit specificity in hemopoiesis. Immunol Rev 2012; 246(1):272–285.
[4] Bottero V, Withoff S, Verma IM. NF-kappaB and the regulation of hematopoiesis. Cell Death Differ 2006; 13(5):785–797.
[5] Coope HJ, et al. CD40 regulates the processing of NF-κB2 p100 to p52. EMBO J 2002; 21:5375–5385.
[6] 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.
[7] Vallabhapurapu S, Karin M. Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol 2009; 27:693–733.
[8] Staudt LM. Oncogenic activation of NF-kappaB. Cold Spring Harb Perspect Biol 2010; 2:a000109.
[9] 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.
[10] 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.
[11] 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.
[12] Woyach JA, Johnson AJ, Byrd JC. The B-cell receptor signaling pathway as a therapeutic target in CLL. Blood 2012; 120:1175-84.
[13] Honigberg, L.A. et al. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. Proc. Natl. Acad. Sci 2010; 107:13075–13080.
[14] Ibrutinib: an evidence-based review of its potential in the treatment of advanced chronic lymphocytic leukemia. Core Evid 2013; 8:37–45.
[15] 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.
[16] 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.
[17] He JQ, Saha SK, Kang JR, Zarnegar B, Cheng G. Specificity of TRAF3 in its negative regulation of the noncanonical NF-kappa B pathway. J Biol Chem 2007; 282(6):3688-3694.
[18] He L, Grammer AC, Wu X, Lipsky PE. TRAF3 forms heterotrimers with TRAF2 and modulates its ability to mediate NF-kappaB activation. J Biol Chem 2004; 279(53):55855-55865.

Attended seminars

1. 20/10/2014 15:00 Dr. Famà Rosella “The Krüppel-like factor 2 transcription factor is a novel tumor suppressor gene recurrently mutated in Splenic Marginal Zone Lymphoma”
2. 01/10/2014 14:00 Dr. Ciardullo Carmela “Clonal evolution andclinical relevance of subclonal mutations in chronic lymphocyticleukemia”
3. 08-22/09/2014 Prof. Steven R. Ellis “The Borghese Sessions”

4. 15/07/2014 14.30-16:00 Prof. Antonia Follenzi "Applicazioni terapia genica''
5. 30/06/2014 14:00-16:00 Dr. Diego Cotella "The C-value paradox, junk DNA and ENCODE"
6. 26/06/2014 14:00 Prof. Gianni Del Sal "Disarming mutant P53 in cancer"
7. 19/06/2014 12:00-13.30 Prof. Antonia Follenzi ''Terapia genica''
8. 12/06/2014 14:00 Prof. Gianni Cesareni "Metformin rewires the signaling network of breast cancer cells and changes their sensitivity to growth and apoptotic stimuli"
9. 11/06/14 14:00 Prof. Fabrizio Loreni "Ribosome alteration in cancer: effect or cause?"
10. 09/06/2014 14:00 Dr. Iacopo Baussano "Assessment of cervical cancer control in Rwanda and Bhutan''
11. 12/05/2014 9:30-15:30 MiniMACS® Simposio "Il ruolo emergente delle vescicole extracellulari in fisiopatologia: da mediatori cellulari a biomarker

Partecipations to conferences

19th Congress of European Hematology Association (EHA). June 12-15 2014, Milan