Biology

172 PROGNOSTIC VALUE OF MYELOID IMPAIRMENT IN HODGKIN'S LYMPHOMA

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Background: In Hodgkin Lymphoma (HL) the interim PET (after 2 cycles of ABVD chemotherapy) is the most important prognostic factor and it is probably linked to the persistence of the reactive microenvironment that promotes tumor cells survival. CD68+ tumor associated macrophages (TAM) are increased in cases with poor prognosis, thus to be proposed as additional prognostic marker at diagnosis of HL. Their progenitors circulating in peripheral blood are well known in solid tumors as Myeloid Derived Suppressor Cells (MDSC) for their ability to suppress T-cell immune responses. In mice, MDSC are broadly defined as being GR1+CD11b+ cells capable of suppressing antigen-specific or nonspecific T cell activation, but there is no accord on the correspondent phenotype in humans.

Patients and Methods: We evaluated by flow cytometry circulating levels of immature im-MDSC (CD11b+, CD13+, CD14-, CD34+, CD45+) in peripheral blood of 37 HL patients at diagnosis and after 2 cycles of chemotherapy. We correlated MDSC to clinical findings included interim-PET response and treatment outcome, and T-cell subpopulations, including Treg (CD4+CD25+FoxP3+).

Results: We found that at diagnosis HL patients have higher levels of im-MDSC, when compared to matched for sex and age healthy controls (p=0.0001) and these cells return to normal values within the first 2 cycles of chemotherapy in five evaluated responders. Absolute number of im-MDSC was positively correlated to Treg absolute count (r = 0,95, p 0,014), in accord to the biologic observation that MDSC are able to expand Treg compartment, but not with other T cell subpopulations. Conversely, no correlation was found with markers of inflammation (ferritin, ESR, C-RP, fibrinogen) tumor burden (stage, IPS, presence of bulky disease) and SUV-PET at diagnosis. Ten patients out of 37 (27%) had increased im-MDSC count at diagnosis (>4.5 cells/μL), with documented positive interim-PET or progression/relapse of disease for 6 of them. Three out of four patients with a positive interim-PET had increased im-MDSC count at diagnosis. The ROC-curve to predict the outcome of interim-PET based on im-MDSC cell count at diagnosis had area=0.96, p=0.0003. PFS in patients with im-MDSC>4.5 cells/μL were comparable to those interim-PET positive (p=0.57) and significantly worse than im-MDSC<4.5 cells/μL (p=0.002).

Conclusion: im-MDSC 1) are increased in peripheral blood of HL patients at diagnosis and decreased after effective treatment 2) correlate with interim PET 3) have a strong prognostic value. They could represent an earlier and more easily accessible prognostic factor than interim-PET and an attractive therapeutic target to improve HL immunotherapy.

173 LACK OF ASSOCIATION OF TUMOR-ASSOCIATED MACROPHAGES WITH CLINICAL OUTCOME IN PATIENTS WITH CLASSICAL HODGKIN LYMPHOMA

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Background: A recent study demonstrated that an increased number of CD68+ macrophages was correlated with primary treatment failure, and shortened progression free survival (PFS) and disease-specific survival (DSS) in patients with classical Hodgkin Lymphoma (cHL).

Patients and Methods: The aim of the present study was to verify the relationship between the number of CD68+ and CD163+ macrophages with clinical outcomes in a cohort of 265 well-characterized patients with cHL treated uniformly with the ABVD chemotherapy regimen. Two pairs of hematopathologists performed independent pathological evaluations of tissue microarray slides.

Results: There were no associations between clinical characteristics and the expression of CD68 or CD163. The expression of CD68 or CD163 was not associated with either the progression-free survival or the disease-specific survival.

Conclusion: CD68 and CD163 expression require further evaluation before their use can be recommended for prognostic stratification of patients with classical Hodgkin's lymphoma.

174 PTPN1 DEPLETION AND ITS CONTRIBUTION TO THE MALIGNANCY OF HRS-CELLS OF CLASSICAL HODGKIN LYMPHOMA.

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Introduction: The Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signalling pathway is constitutively active in at least a subset of B-cell derived lymphomas, e.g., primary mediastinal B-cell lymphoma (PMBL; 2) and classical Hodgkin's lymphoma (cHL; 3), and was shown being associated with cell survival, immune escape and tumor angiogenesis. In PMBL, this deregulation is due to function-impairing mutations of negative-regulatory proteins like the Silencer of Cytokine Signaling 1 (SOCS-1), enhancing phosphorylation and activation of JAK2 (2).

Methods and results: Our results point to a new mechanism of deregulated JAK2/STAT signalling in cHL. In U-HO1, a novel cell line derived from a primary refractory Hodgkin lymphoma (1), we observed that activity of the non-receptor Protein Tyrosine Phosphatase N1 (PTPN1) is completely lost. In addition, it varied considerably in the other 6 cHL-cell lines, with very low levels of PTPN1 activity in SUP-HD1. In both, U-HO1 and SUP-HD1, PTPN1 cDNA is strikingly truncated by an abnormal exon skipping mechanism. Complementation of the functional PTPN1 knockout by stable expression of PTPN1 in U-HO1, results in abolishing nuclear expression of STAT5, a very slow cell proliferation rate with an elongated generation time and higher levels of apoptosis. A complete lack of PTPN1 was also found in the parental tumor of U-HO1, being restricted to the Reed-Sternberg (HRS) cells.

Conclusion: In conclusion, we could show that inactivation of PTPN1 contributes to the malignant phenotype of cHL by sustaining STAT5 activity, preventing senescence and evasion from apoptosis of the neoplastic multinucleated HRS cells. This selection advantage is a notorious feature in this type of tumor cells. Nevertheless, PTPN1 can be a potential tumor suppressor in cHL that might be a marker for clinical outcome prognosis and for the recognition of early-stage disease patients with a high risk of recidivism.

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175 A NOVEL UBIQUITIN-BASED REGULATION OF THE MTOR/AKT SIGNALING PATHWAY UNDERLIES THE FORMATION OF B-CELL LYMPHOMA IN UCHL1 TRANSGENIC MICE

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Background: UCH-L1, a de-ubiquitinase (DUB) that is normally restricted to neural tissues, is frequently over-expressed in primary B-cell lymphomas across the histological spectrum. We have generated Uchl1 transgenic mice and found them to be highly prone to the development of B-cell lymphoma. Mechanistically, we observe that UCH-L1 boosts signaling of the Akt pathway in vitro and in vivo, though the molecular basis of this effect is unknown. Akt is phosphorylated by the kinases PDK1 and mTOR. The activity and substrates of mTOR depend on its association into two distinct complexes, with mTORC1 (mTOR/raptor) acting as a regulator of cap-dependent mRNA translation, and mTORC2 (mTOR/rictor/SIN1) being an essential kinase responsible for phosphorylating AktSer473.

Materials and Methods: We performed biochemical studies using primary samples from Uchl1 transgenic and knockout mice, as well as malignant human B-cell lines (KMS-28, myeloma), transduced with short hairpin RNAs. We use immunoblotting, and immunoprecipitation to study the assembly and signaling of the mTOR/Akt pathway.

Results: Depletion of UCH-L1 in KMS-28 cells leads to a dramatic decline in Akt phosphorylation that is not due to changes in the levels of PDK1, mTOR, or any mTOR complex component. We observe a striking increase, however, in the association of mTOR with raptor, and a reciprocal decline in the mTORC2 subunit rictor, suggesting that UCH-L1 promotes mTORC2 assembly. This is verified when we introduce UCH-L1 into HeLa cells and observe an increase in the co-precipitation of rictor with mTOR, with a corresponding decrease in the recovery of raptor. This effect is entirely dependent on DUB activity, as expression of a catalytic mutant has no effect on complex assembly. We find that UCH-L1 forms a novel complex containing the ubiquitin-ligase DDB1. The formation of this complex leads to the displacement of DDB1 from raptor. The DDB1-raptor complex is required for mTORC1 signaling. Consistent with these results, we find that mTORC1 activity is greatly reduced in cells with high levels of UCH-L1, and enhanced in the brains of Uchl1 null mice.

Conclusion: These data directly implicate UCH-L1 as a novel regulator of mTOR activity through a mechanism that requires its de-ubiquitinase activity. UCH-L1 produces a dramatic re-organization of mTOR complexes with a resulting increase in Akt phosphorylation. High levels of UCH-L1 shift cells away from the mTORC1 pathway that is targeted by rapamycin, suggesting that cancers with high levels of UCH-L1 may benefit most from inhibitors that instead target the mTORC2 pathway.

176 SEROLOGICAL PROTEOME ANALYSIS (SERPA) FOR THE IDENTIFICATION OF NOVEL TUMOR-ASSOCIATED ANTIGENS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: In this study a serological proteome analysis (SERPA) was performed to identify novel tumor associated antigens (Ag) capable of eliciting humoral immune responses in patients with chronic lymphocytic leukemia (CLL).

Material and Methods: Twenty-one untreated CLL patients were included in the study. Proteins extracted from leukemic cells were separated by 2-D electrophoresis (2-DE) and transferred onto membranes by electroblotting to obtain 21 proteomic maps. Sera from CLL patients were screened individually by Western Blot (WB) for antibody-based reactivity against autologous proteins in corresponding maps. Seven out of 21 CLL maps were also probed with control sera collected from 7 healthy donors (HD). Ag were identified by MALDI-TOF mass spectrometry (MS).

T cells isolated from the PB of 3 CLL patients with anti-ENOA antibodies were stimulated with autologous ENOA-pulsed and control dendritic cells (DC), and evaluated by IFNγ ELISPOT assay.

Results: Sixteen out of 21 CLL sera (76%) showed at least one immunoreactivity and produced an overall number of 45 Ag spots. By contrast, sera from HD were significantly less reactive ($p<.03$) and produced only a total of 3 Ag spots. Eleven out of 16 (69%) reactive CLL sera recognized from 2 to 6 different Ag. The reactivity rate and number of WB spots did not correlate with main parameters of clinical outcome. Sera from 48% CLL patients exhibited reactivity against a protein which was identified by MS as α-Enolase (ENOA). ENOA recognition was CLL specific since none of the sera from HD showed reactivity against this protein. Interestingly, ENOA was recognized from sera of 7 out of 12 mutated patients (58%), but only from sera of 2 out of 8 unmutated patients (25%).

CLL-derived ENOA-pulsed DC stimulated autologous T cells to secrete IFNγ. This response was ENOA-specific because it was not induced by unpulsed DC or DC pulsed with an irrelevant protein, and also CLL-specific because IFNγ release was not induced when T cells from a HD were stimulated with autologous ENOA-pulsed DC.

Conclusion: Our results indicate that ENOA is capable of eliciting CLL-specific humoral and cellular immune responses. Therefore, ENOA can be considered a promising biomarker and a potential target for immunotherapeutic approaches in CLL.

177 CLL CELLS INDUCE CLONAL T CELL SKEWING AND SUBSETSHIFTING IN THE MURINE TCL1 TRANSGENIC CHRONIC LYMPHOCYTIC LEUKEMIA MODEL

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Introduction: Chronic lymphocytic leukaemia (CLL) thrives in relevant association with microenvironmental factors. A number of abberations in the T cell compartment have been described in human CLL, from higher global numbers of T cells present in CLL to altered gene expression profiles in CLL-derived T cells compared to healthy donors. We previously showed skewed T cell subsets in CLL and were able to predict clinical behaviour based on T cell markers. An analysis of complex intercellular relations, however, is experimentally limited in human samples and some insights might be facilitated by the use of murine models of disease.

Material and Methods: We use Eμ-tcl1 transgenic (tg) CLL mice to investigate the T cell compartment. Backcrossing the mice to a pure C57BL/6 genetic background to perform transplantation experiments without a HLA barrier we could indeed reliably engraft Tcl1 tg CLL cells into congenic wildtype C57BL/6 mice without immunosuppression.

Results: First, we followed the T cell compartment throughout spontaneous development of CLL in the mice. We found a prominent shift from a mainly naive T cell compartment in wild type and young Tcl-1 tg mice to predominance of antigen experienced central memory CD4 and CD8 T cell subsets in mice with CLL. Indeed, we find a correlation between the proportion of CLL cells and the skewing of the T cells in affected tissues. Since this was indirect evidence that CLL may actively instruct the changes in the T cells, we used the transplant model to determine the effect of CLL cells on a wild type T cell repertoire. We found a rapid shift from naive to experienced T cells in the transplanted mice and show, in CDR3 spectratyping analysis, that the resulting T cell repertoire is clonally skewed. Because of the possibility of expansion of contaminating donor T cell from the transplant in the recipient, we transplanted CLL into GFP-gene marked recipients and show that the CLL cells redirect naive recipient T cells, rather than help expand clones from the donor with CLL disease.

Conclusions: We validate a backcrossed Tcl1 tg mouse model for modelling T cell changes observed in human CLL and present a transplantation model suitable to investigate drugs with important influence on the T cell micromilieu (such as the ''imids''). In addition, we present the first evidence in the murine model, that changes in the T cell compartment can effectively be induced by CLL and that the mechanism most likely includes an antigenic selection of T cells.

178 PEPTIDES COMPETE WITH HUMAN HERPESVIRUS 8K1-FAS COMPLEXES AND RESTORE APOPTOSIS.

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Background: The long-term expression of human herpesvirus 8 (HHV-8) K1 produces hyperplasia of lymph nodes, splenomegaly and lymphomas in mice. The immunoreceptor tyrosine-based activation motif (ITAM) of K1 was shown to activate Akt. We have recently shown that K1 suppresses Fas-mediated apoptosis directly through its extracellular immunoglobulin-like domain. We thus hypothesized that K1-expressing mice develop hyperplasia and lymphomas driven by altered Fas signaling.

Methods: Fas-mediated apoptosis was analyzed by incubation of K1 transgenic mice-derived cells in the presence of agonistic anti-Fas antibody (Jo2) and monitoring of cells for morphologic evidence of apoptosis. Mouse tissues was analyzed after staining with hematoxylin and eosin staining and with anti-kappa or lambda antibodies. KI-Fas complexes were monitored in immunoprecipitation/immunoblotting (IP/IB) analysis in the presence of K1-derived peptides and anti-Fas antibody.

Results: Examination of mice expressing K1 via a ubiquitous promoter showed that K1 transgenic mice (n=10) had 90% developed lymphoid hyperplasia (at least 3 lymph nodes >3 mm) and 60% developed lymphomas after 18 months, while all (26) control nontransgenic mice remained free of lymph node hyperplasia and lymphoma. The spleens of 78% of K1 mice were enlarged at 18 months and were on average 3.5 times heavier than spleens of non-K1 transgenic control mice. Anti-kappa and anti-lambda light chain antibodies revealed the presence of monoclonal foci in 3 out of 3 K1 mice (average 6 foci per single section of spleen), but no foci were present in 4 control non-transgenic mice. To test whether expression of K1 protein confers resistant to Fas-mediated apoptosis, splenic cells of 6-month-old K1 mice (n=3) and matched controls (n=3) were incubated with 50 ng/mL of agonistic anti-Fas antibody Jo2, which produce apoptosis in 11±0.6% of splenocytes from K1 mice versus 50±6%, ($P<0.005$) of control splenocytes. Of mice inoculated with a lethal dose of Jo2 antibody, 3 out of 12 K1 transgenic (30%) and 13 out of 22 control mice (60%) died ($P<0.05$). Overexpression of an Ig domain-containing protein CD79b competed with K1-Fas binding in a dose-dependent manner. Two 20-amino acid peptides (N251, N253) representing the Ig domain of K1 disrupted K1-Fas binding in (IP/IB) analysis and enhance Fas-mediated apoptosis.

Conclusion: These results suggest a key role of HHV-8 K1 in lymphoid hyperplasia and lymphoma and for K1 in direct modulation of Fas during apoptosis.

179 GENE EXPRESSION MICROARRAY ANALYSIS OF MANTLE CELL LYMPHOMA BY CDNA MICROARRAY COMBINED WITH LASER MICRODISSECTION: THE WNT SIGNALING PATHWAY MAY PLAY A CENTRAL ROLE IN LYMPHOMAGENESIS WITH THE T(11;14) TRANSLOCATION, WHILE THE SPECIFIC MITOTIC REGULATORS FACILITATE THE TRANSFORMATION INTO THE AGGRESSIVE FORM.

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Introduction/background: For an accurate understanding of mantle cell lymphoma (MCL), molecular behaviors could be staged into two major events: lymphomagenesis

with the t(11:14) translocation (*initial event*), and variable secondary genomic alterations occurring over time for evolution into the aggressive form (aMCL) (*evolutional event*). Unfortunately, it is still unknown which genes contribute to which events.

Material and Methods: We performed cDNA microarray experiments using frozen tissues of lymph node biopsies. In order to identify the genes and their contributions to the events respectively, we posited two stepwise morphological grades for classical MCL (cMCL): mantle zone cMCL and nodular cMCL. For evaluation of the *initial event*, we compared samples from the tumor cells of mantle zone cMCL (n=4) with those from normal mantle zone B-lymphocytes (n=4), which were derived from selected specimens by means of laser microdissection (LMD). To compensate for individual differences, the evaluation of the *evolutional event* was performed in combination with that of two MCL cases with transformational morphology, that is, intermediate MCL (iMCL), with the two features (classical and aggressive) morphologically involving the same site. For the *evolutional event,* total RNAs from whole tumor tissue samples, nodular cMCL (n=4) and aMCL (n=5) were used for the analysis, while we also compared the microdissected classical area with the aggressive area in iMCL obtained by means of LMD. Finally, we selected the overlapping genes within them.

Results: *Evolutional event*. We identified 60 overlapping genes, which showed significant differences in their evolutional event. IPA (Ingenuity pathway analysis) clearly and in detail showed the network composed of p53 and several of its interaction partners. Most of these genes were considered to be of interest because of their known function as mitotic regulators, which mediate cell cycle progression during the G2/M transition. CDC2, FOXM1, and BIRC5 in particular interact with many other highly expressed genes in aMCL, which suggests that they may play a critical role in transformational events in coexistence with p53.

Initial event. We selected 1,538 genes, which showed significant differences with a P-value of <0.05. For further analysis, we focused on the WNT signaling pathway, because IPA analysis revealed that a significant number of these genes (n=23) belonged to this category (P=0.016). In addition, WNT3 and LRP5 were not only genes with a high average increase (2.65- and 1.04-fold, respectively), but also genes which are reportedly highly expressed in MCL. Interestingly, none of the genes associated with the WNT signaling pathway were significantly changed in the evolutional event. cDNA microarray showed that β-catenin was not significantly up-regulated in the initial event, but the immunohistochemical study revealed that most of the mantle zone cMCLs showed nuclear localization of β-catenin with high levels of cytoplasmic WNT3 staining. On the other hand, the reactive mantle zone B-cells were positive for β-catenin in the cytoplasm with low levels of WNT3 staining.

Conclusions: The WNT signaling pathway may play a central role in lymphomagenesis with the t(11:14) translocation, while the specific mitotic regulators facilitate the transformation into the aggressive form.

180 THE MIRNA-17~92 CLUSTER MEDIATES CHEMORESISTANCE AND ENHANCES TUMOR GROWTH IN MANTLE CELL LYMPHOMA VIA PI3K/AKT PATHWAY ACTIVATION

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Introduction: The median survival of patients with mantle cell lymphoma (MCL) ranges from three to five years with current chemotherapeutic regimens. A common secondary genomic alteration detected in MCL is chromosome 13q31-q32 gain/amplification, which targets a microRNA (miRNA) cluster, miR-17~92. It has been shown that overexpression of miR-17~92 accelerates MYC-induced lymphomagenesis in mice. However, the role of this miRNA cluster in MCL is still elusive.

Patients and Methods: Gene expression profiling (GEP) was performed on 82 patients with MCL using Affymetrix HG-U133 plus 2.0 arrays. The expression level of the *C13orf25* gene was determined and correlated with clinical outcomes. MCL cell lines with ectopic expression or conditional knockdown of miR-17~92 were constructed and tumor cell proliferation was assessed. Protein and phosphoprotein levels were also determined by immunoblotting. Xenograft MCL model was generated by inoculating MCL cells with or without the decoy or ''sponge'' construct into CB-17/SCID mice and the tumor growth was evaluated.

Results: We found that high level expression of *C13orf25*, the primary transcript from which these microRNAs are processed, was associated with poorer survival in patients with MCL (p=0.021). We demonstrated that the phosphatases PTEN and PHLPP2, important negative regulators of the PI3K/AKT pathway, were both direct targets of miR-17~92 miRNAs and down-modulated in MCL cells with overexpression of the miR-17~92 cluster. Overexpression of miR-17~92 activated the PI3K/AKT pathway and inhibited chemotherapy-induced apoptosis in MCL cell lines. Conversely, inhibition of miR-17~92 expression using the decoy or ''sponge'' construct suppressed the PI3K/AKT pathway and inhibited tumor cell growth in MCL cell lines and in xenograft MCL mouse model. Furthermore, Inhibition of miR-17~92 expression enhanced tumor cell sensitivity to standard chemotherapy in xenograft tumors.

Conclusion: Overexpression of miR-17~92 down-modulates multiple proteins involved in PI3K/AKT signaling and apoptosis, and inhibition of miR-17~92 inactivate PI3K/AKT signaling and inhibit MCL tumor growth. Targeting the miR-17~92 cluster may therefore provide a novel therapeutic approach for patients with MCL.

181 CLINICAL OUTCOME OF PATIENTS WITH NON-HODGKIN'S LYMPHOMA MAY BE INFLUENCED BY AN INHERITED FUNCTIONAL POLYMORPHISM IN THE INTERLEUKIN 12 GENE

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Introduction: The immunologic interaction between the cancer and the host may be influenced by cancer factors and host factors. We have been interested in inherited polymorphisms in key genes that regulate the innate or adaptive immune response and how these may influence the immunologic response to the cancer and clinical outcome. Single nucleotide polymorphisms (SNPs) were selected based on published data with risk of cancer, or cancer survival outcomes.

Materials and Methods: This report focuses on analysis of 192 patients including 124 patients with diagnosed with aggressive and 68 patients with low grade non-Hodgkins lymphoma (NHL). Analysed genes included; IL8RB (rs1126580), P2RX7 (rs3751143), IL1A (rs1800587), IL4R (rs2107356), IL12B (rs3212227), FCGR3A (F158V). SNP genotyping was performed with real-time PCR on a Rotor Gene Q instrument. Allele specific PCR assays were designed to produce clearly distinguishable melt peaks for each genotype. PCR reactions were set up with the aid of a Qiagility robot for rapid, accurate processing.

Results:

	GENOTYPES	NUMBER	%	DEATHS	%	MEDIAN OVERALL SURVIVAL (mon)	*p*	IPI < 1	*p*
		n= 124							
IL12B aggressive only	AA/AC	114	92%	55	48%	44.9	0.1895	26%	*0.0012*
	CC	10	8%	2	20%	Undefined		80%	
		n=194							
IL12B including low grades	AA/AC	176	92%	76	43%	51.7049	0.0509		
	CC	16	8%	2	13%	Undefined			

Table1. Results of genotyping of IL12B.

No statistically significant relationships between genotype and overall survival were found for any of the six SNPs when only 124 aggressive patients were analyzed, but the IL12B variant CC that has been associated with higher levels of secreted IL-12, was associated with fewer deaths and extended long term overall survival. Also an IPI score of 0-1 was significantly more frequent in the IL12B CC group. We did an additional analysis of the IL12B polymorphism including 68 low grade NHL patients for a total of 192 subjects. In this case, the p value reached 0.0509. Multivariate analyses and more detailed modeling of combinations of these polymorphisms will be presented.

Conclusions: Patients with aggressive NHL with IL12B CC may have enhanced overall survival. We postulate that the immune response of these patients may have a more effective Th1 and cytotoxic T lymphocyte polarization based upon the inherited predisposition to increased IL12 responses. We are continuing our analysis of additional polymorphisms and additional study populations.

182 PROGNOSTIC IMPACT OF TUMOR-ASSOCIATED MACROPHAGES ON SURVIVAL OF DIFFUSE LARGE B-CELL LYMPHOMA PATIENTS TREATED WITH HIGH-DOSE CHEMOTHERAPY SUPPORTED WITH AUTOLOGOUS STEM CELL TRANSPLANTATION

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Background: Non-malignant cells of the tumour microenvironment have been shown to contribute to prognosis in diffuse large B-cell lymphoma (DLBCL) patients. Here, we have examined the association of tumour-associated macrophage (TAM), mast cell (MC) and microvessel content with the outcome

of DLBCL patients treated with high dose chemotherapy supported with autologous stem cell transplantation (ASCT).

Patients: Tumor samples from 52 DLBCL patients, with primary (n=36) or relapsed (n=33) disease, treated with high-dose chemotherapy (HD-CT) supported with ASCT, were immunohistochemically evaluated for CD68, mast cell tryptase and CD31 expression to detect TAMs, MCs and microvessels, respectively. Data were correlated with survival parameters. In this patient cohort, a median follow-up was 86 months, and the 5-year relapse free survival (RFS) was 57% and overall survival (OS) was 55%.

Results: In Cox univariate analysis, TAM content had prognostic impact on RFS ($p = 0.027$), and on OS ($p = 0.025$). In addition, when TAM number was adjusted for IPI, it remained as significant prognostic factor ($p = 0,037$). According to Kaplan Meier estimates, the patients with low TAM content (< median) had better 5-year RFS and OS rates when compared to patients with high TAM number (72% versus 42%, $p = 0.04$ for RFS and 69% versus 41%, $p = 0.038$ for OS). No correlation was found between MC or microvessel content and survival parameters.

Conclusions: The data suggest that high TAM score is associated with unfavourable prognosis in DLBCL-patients treated with HD-CT supported with ASCT.

183 P53 PATHWAY ANALYSIS IN PEDIATRIC BURKITT LYMPHOMA IDENTIFIES A SUBSET OF CASES WITH HDM4 LOCUS GAIN, INCREASED HDM4 MRNA, AND LACK OF OTHER P53 PATHWAY ABNORMALITIES

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Background: P53 pathway abnormalities are common in tumors and include deletion or mutation of the *TP53* locus and overexpression of the p53 pathway inhibitors HDM4 and MDM2, which can result from copy number gains of their loci. *TP53* mutations and MDM2 overexpression have been described in pediatric Burkitt lymphoma (pBL), but the expression patterns of HDM4 in pBL have not been reported. In order to investigate p53 pathway disruption in pBL, we utilized high resolution genome wide copy number analysis, *TP53* mutation screening, RT-PCR, and immunohistochemistry (IHC) to correlate gene copy number change at *TP53*, *MDM2*, and *HDM4*; *TP53* mutations; *MDM2* and *HDM4* transcript levels; and protein expression of p53, MDM2, HDM4, and p21.

Materials and Methods: Tumor (n=30) and germline reference (n=25, from normal staging bone marrows) DNA was isolated from formalin-fixed, paraffin-embedded (FFPE) tissue samples and submitted for Molecular Inversion Probe (MIP 330K Cancer Panel, Affymetrix) assay. Data were analyzed for genome-wide copy number, loss of heterozygosity (LOH), and *TP53* mutations. *TP53* mutations predicted by MIP analysis were amplified by PCR and sequenced. Relative levels of mRNA (also from FFPE) for *MDM2* and *HDM4* were assayed by RT-PCR.MDM2, HDM4, p53, and p21 protein were assessed by IHC.

Results: Genome wide copy number analysis and mutation screening was successfully performed on 26/30 samples. We identified *TP53* genetic changes in 8/26 (31%) cases including deletions (3/26), LOH (3/26), and mutations (5/26). Mutations were verified in all 5 cases by sequencing. Gains of 1q32 that include the *HDM4* locus were seen in 6/26 patients (23%) while specific amplification or deletion of the *MDM2* 12q15 locus was not seen. *HDM4* transcripts varied over a 4-fold range among the 24 tumor samples tested and were higher in samples with 1q32 gain (4/5 with 1q32 gain in the top quartile of transcript expression vs. 2/17 without gain, $p<0.003$). *MDM2* mRNA levels showed a 2-4-fold increase (compared to reactive lymph node) with one sample showing a 16-fold increase. HDM4 protein was expressed in 28/28 tumors with intensity slightly lower than reactive germinal center B-cells in most cases. MDM2 protein was weakly expressed in 10/28 (35%). P21 was negative in all cases (0/28) with appropriate positive controls.p53 protein was expressed in 15/28 (54%) cases, including 7/8 with *TP53* genetic alterations. Cases with *HDM4* gain did not express p53 protein (0/6 vs. 16/20 without the gain, $p<0.0005$). Cases in the top quartile of *HDM4* mRNA were also less likely to express p53 protein (2/6 vs. 13/16, $p=0.032$).

Conclusions: We report using the MIP assay for genome wide copy number analysis and *TP53* mutation screening, along with RT-PCR and IHC, to demonstrate deregulation of the p53 pathway in the majority of our pBL cases. All mutations identified by the MIP screen were confirmed by sequencing. Although HDM4 protein appears uniformly expressed in pBL by IHC, transcript levels vary and are significantly associated with *HDM4* locus gain. In addition, cases with locus gain and/or higher levels of *HDM4* transcripts tended to lack *TP53* genetic alterations and p53 protein, a surrogate marker of p53 mutation, which suggests that *HDM4* overexpression may be the primary mechanism of p53 pathway disruption in some cases of pBL.

184 INTERPLAY BETWEEN THE VIRAL PRODUCT EPSTEIN-BARR NUCLEAR ANTIGEN-1 AND THE CELLULAR MICRORNA-127 IN BURKITT LYMHPHOMA

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Introduction: Epstein Barr Virus (EBV) is a γ-herpesvirus that infects more than 90% of the human population. Although EBV persists in its latent form, this virus is also associated with the 15% percent of human cancer, such as Burkitt Lymphoma (BL). However, there is still no satisfactory explanation of how EBV participates in its pathogenesis. One striking feature of EBV-pos BLs is their unique pattern of viral latent protein expression, which is restricted to EBV-encoded nuclear antigen 1 (EBNA1). Recently, new insights on the interplay between viruses and microRNAs (miRNA) has been proposed, thereby regulating cellular gene expression. We previously demonstrated specific up-regulation of hsa-miR-127 in EBV-pos BLs. Here, we investigate the mechanism of hsa-miR-127 regulation possibly mediated by EBNA1 and the effects of their combinatorial expression on the impairment of B cell function.

Patients and Methods: C57/B6 mice were immunised with sheep red blood cells (SRBC) and splenic B220+Fas- and FashighB220+ cells were sorted using a FACSAria. A human lymphoblastoid cell line LCL, EBV-negative BL cell line Ramos and human memory B cells were transfected with EBNA1 vector. Luciferase assay was performed using pGL3 containing the miR127 promoter and pcDNA3-EBNA1. Human memory B cells were co-transfected with miR127 and EBNA1. The relative expression of both hsa-miR-127 and B-cell markers was evaluated by qRT-PCR.

Results: Firstly, we confirmed a regulation of miR127 during normal immune function in a murine model. Furthermore, we showed that hsa-miR-127 expression increases after ectopic expression of EBNA1 in LCL, Ramos and human memory B cells. In addition, the co-expression of hsa-miR-127 and EBNA1 in human memory B cells, the postulated normal counterpart of EBV-pos BL, provided evidence of an impairment of B-cell markers (BLIMP-1, XBP-1, IRF-4 and BCL-6). Finally, we demonstrated that EBNA1 activates a luciferase construct driven by miR127 promoter.

Conclusions: On the basis of our results, we speculate that EBNA1 is involved on the miR127 activation, probably occurring in a memory B cell, suggesting a novel mechanism of miRNA regulation by viral products. The ability of hsa-miR-127 to modulate multiple genes of B-cell impairment seems to be realized in concert with EBNA1. A good understanding of these mechanisms will help to clarify the complex link between the regulatory networks of the host and pathogen and design more specific treatments on EBV-associated malignancies.

185 COMPARATIVE GENOMIC IDENTIFICATION OF SIGNALING PATHWAYS AND TARGETS IN PEDIATRIC BURKITT LYMPHOMA (PBL)

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Background: The prognosis of PBL has dramatically improved over the past 25 years (≥ 80% 5 yrs EFS) (Cairo et al BJH 2009). This success, however, has come at the cost of the high toxicity with intensive chemotherapy. A need to identify less toxic targeted therapy exists. MAP kinases (MAPK), protein tyrosine kinases (PTK) and protein tyrosine phosphatase (PTP) inhibitors/siRNA may be utilized to treat PBL. We seek to identify PBL pathways and potential drug targets for patients with PBL.

Methods: PBL data from 3 different research groups were used; i.e. COG ANHL01P1 by our group (n=11), GSE10172 and GSE4475 (Klapper et al; n=16), and GSE4732 (Dave/Staudt et al; n=15). For normalized comparison, ANHL01P1 samples were validated by building a prediction model with Support Vector Machines using Klapper PBL as training database. RNAs were subjected to microarray studies (Affymetrix U133A_2) and analyzed by Agilent GeneSpring or Partek. Identified PBL genes were analyzed by Ingenuity Pathways Analysis. One-way ANOVA followed by Tukey test was used. Immunostaining was performed using monoclonal antibodies to TUBB2C and IRAK1. Results. 1565 genes were identified ($p<0.05$), among which 376 genes were similarly expressed.cMYC (27.0F) & TUBB2C (39.7F) were highly expressed, consistent with the BL characteristics. These genes are involved in TOLL-like receptor signaling ($p<0.01$), including IRAK1 (22.9F; a serine/threonine kinase), NFKBIA (15.3F); JAK-STAT signaling ($p<0.01$), including JAK1 (25.0F; a PTK), IFNGRI (14.5F), IFNGR2 (12.0F), STAT1 (9.2F), PTPN11 (25.6F), PIM1 (6.4F); and MAPK signaling ($p<0.01$) including MAP2K1 (11.8F), MAPK9 (6.9F), RAF1 (9.2F; a MAP3K). Fold changes of qRT-PCR results were consistent with genomic data. By immunohistochemistry, 28/30 PBL were positive for TUBB2C protein with variable intensity while 3/10 showed weak cytoplasmic reactivity for IRAK1.

Conclusions: Taken together, JAK-STAT and MAPK signaling pathways play important roles in PBL. The interaction of IFN-gamma with IFNGRI & IFNGR2 stimulates the activation of JAK1 and PTPN11, which in turn, regulates the MAPK pathway. The significance of TOLL receptor signaling may contribute to a subgroup of PBL. JAK1, PTPN11, & RAF1 may be potential targets in PBL.

186 THE DISTINCT MOLECULAR SIGNATURE OF HEPATOSPLENIC T-CELL LYMPHOMA (HSTL) IDENTIFIES ONCOGENIC PATHWAYS WITH POTENTIAL THERAPEUTIC RELEVANCE

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Background: HSTL is a rare entity characterized by an infiltration of bone marrow, spleen and liver tissues by neoplastic gammadelta (gd) -more rarely alphabeta (ab)- T cells. Its pathogenesis is poorly understood. Our purpose was to identify the molecular signature of HSTL and explore molecular pathways implicated in its pathogenesis.

Methods: Gene expression profiling and array CGH analysis of 10 HSTL samples (7gd, 3ab), 1 HSTL cell line (DERL2), 2 normal gd samples together with 16 peripheral T-cell lymphoma not otherwise specified (PTCL,NOS) and 7 nasal NK/T cell lymphomas were performed.

Results: By unsupervised analysis, ab and gdHSTL clustered together remarkably separated from other lymphoma entities. Compared to PTCL, NOS, HSTL overexpresed genes encoding NK-associated molecules, oncogenes (VAV3) and the Sphingosine-1-phosphatase receptor 5 involved in cell trafficking. Compared to normal gd cells, HSTL overexpressed genes encoding NK-cell and multi drug resistance-associated molecules, transcription factors (RHOB), oncogenes (MAFB, FOS, JUN, VAV3) and the tyrosine kinase SYK whereas genes encoding cytotoxic molecules and the tumor suppressor gene AIM1 were among the most downregulated. By immunohistochemistry, SYK was demonstrated on HSTL cells with expression of its phosphorylated form in DERL2 cells by Western blot. Functional studies using a SYK inhibitor revealed a dose dependent increase of apoptotic DERL2 cells suggesting that SYK could be a candidate target for pharmacologic inhibition. Downexpression of AIM1 was validated by qRT-PCR. Methylation analysis of DERL2 genomic DNA treated by bisulfite demonstrated highly methylated CpG islands of AIM1. Genomic profiles confirmed recurrent isochromosome 7q (n=6/9) without alterations at 9q22 and 6q21 containing SYK and AIM1 genes, respectively.

Conclusion: The current study identifies a distinct molecular signature for HSTL and highlights oncogenic pathways which offer rationale for exploring new therapeutic options such as SYK inhibitors. It supports the view of gd and ab HSTL as a single entity.