OncoImmunology

ISSN: (Print) 2162-402X (Online) Journal homepage: http://www.tandfonline.com/loi/koni20

Interleukin 10 (*IL10*) proximal promoter polymorphisms beyond clinical response in classical Hodgkin lymphoma: Exploring the basis for the genetic control of the tumor microenvironment

Gabriela Vera-Lozada, Carolina Minnicelli, Priscilla Segges, Gustavo Stefanoff, Flavia Kristcevic, Joaquin Ezpeleta, Elizabeth Tapia, Gerald Niedobitek, Mário Henrique M. Barros & Rocio Hassan

To cite this article: Gabriela Vera-Lozada, Carolina Minnicelli, Priscilla Segges, Gustavo Stefanoff, Flavia Kristcevic, Joaquin Ezpeleta, Elizabeth Tapia, Gerald Niedobitek, Mário Henrique M. Barros & Rocio Hassan (2018) *Interleukin 10* (*IL10*) proximal promoter polymorphisms beyond clinical response in classical Hodgkin lymphoma: Exploring the basis for the genetic control of the tumor microenvironment, OncoImmunology, 7:5, e1389821, DOI: 10.1080/2162402X.2017.1389821

To link to this article: https://doi.org/10.1080/2162402X.2017.1389821

© 2018 The Author(s). Published with license by Taylor & Francis Group, LLC© Gabriela Vera-Lozada, Carolina Minnicelli, Priscilla Segges, Gustavo Stefanoff, Flavia Kristcevic, Joaquin Ezpeleta, Elizabeth Tapia, Gerald Niedobitek, Mário Henrique M. Barros, and Rocio Hassan

Accepted author version posted online: 31 Oct 2017.
Published online: 29 Mar 2018.

Article views: 246

View supplementary material

Submit your article to this journal

View Crossmark data

Full Terms & Conditions of access and use can be found at
http://www.tandfonline.com/action/journalInformation?journalCode=koni20

ONCOIMMUNOLOGY
2018, VOL. 7, NO. 5, e1389821 (10 pages)
https://doi.org/10.1080/2162402X.2017.1389821

Taylor & Francis
Taylor & Francis Group

ORIGINAL RESEARCH

OPEN ACCESS Check for updates

Interleukin 10 (*IL10*) proximal promoter polymorphisms beyond clinical response in classical Hodgkin lymphoma: Exploring the basis for the genetic control of the tumor microenvironment

Gabriela Vera-Lozada [a], Carolina Minnicelli[a,b], Priscilla Segges[a], Gustavo Stefanoff[c], Flavia Kristcevic[d], Joaquin Ezpeleta[d], Elizabeth Tapia[d], Gerald Niedobitek[e], Mário Henrique M. Barros[e], and Rocio Hassan [a]

[a]Oncovirology Laboratory, Bone Marrow Transplantation Center (CEMO), Department of Clinical Analysis and Toxicology, Instituto Nacional de Câncer (INCA), Rio de Janeiro, Brazil; [b]Universidade Federal do Rio Grande do Norte (UFRN), Natal RN, Brazil; [c]Coordination of Clinical Research, INCA, Rio de Janeiro, Brazil; [d]Centro Internacional Franco Argentino de Ciencias de la Información y de Sistemas (CIFASIS), CONICET, Rosario, Argentina; [e]Institute for Pathology, Unfallkrankenhaus Berlin, Berlin, Germany

ABSTRACT

Interleukin-10 (IL10) is an immune regulatory cytokine. Single nucleotide polymorphisms (SNPs) in IL10 promoter have been associated with prognosis in adult classical Hodgkin lymphoma (cHL). We analyzed IL10 SNPs −1082 and −592 in respect of therapy response, gene expression and tumor microenvironment (TME) composition in 98 pediatric patients with cHL. As confirmatory results, we found that −1082AA/AG; −592CC genotypes and ATA haplotype were associated with unfavourable prognosis: Progression-free survival (PFS) was shorter in −1082AA+AG (72.2%) than in GG patients (100%) (P = 0.024), and in −592AA (50%) and AC (74.2%) vs. CC patients (87.0%) (P = 0.009). In multivariate analysis, the −592CC genotype and the ATA haplotype retained prognostic impact (HR: 0.41, 95% CI 0.2–0.86; P = 0.018, and HR: 3.06 95% CI 1.03–9.12; P = 0.044, respectively). Our analysis further led to some new observations, namely: (1) Low IL10 mRNA expression was associated with −1082GG genotype (P = 0.014); (2) IL10 promoter polymorphisms influence TME composition;−1082GG/−592CC carriers showed low numbers of infiltrating cells expressing MAF transcription factor (20 vs. 78 and 49 vs. 108 cells/mm^2, respectively; P< 0.05); while ATA haplotype (high expression) associated with high numbers of MAF+ cells (P = 0.005). Specifically, −1082GG patients exhibited low percentages of CD68+MAF+ (M2-like) intratumoral macrophages (15.04% vs. 47.26%, P = 0.017). Considering ours as an independent validation cohort, our results give support to the clinical importance of IL10 polymorphisms in the full spectrum of cHL, and advance the concept of genetic control of microenvironment composition as a basis for susceptibility and therapeutic response.

ARTICLE HISTORY
Received 4 March 2017
Revised 3 October 2017
Accepted 4 October 2017

KEYWORDS
cHL; Single Nucleotide Polymorphisms (SNP); survival; tumor microenvironment; MAF; macrophages

Introduction

Interleukin 10 (*IL10*) is an immune regulatory cytokine with non-redundant roles in anti-inflammatory responses,[1] B-cell proliferation,[2] and differentiation of T and B cell subsets.[3,4] In addition, it has important roles in the polarization of specific macrophage subsets, both in infectious conditions and cancer.[5,6]

Inter-individual differences in *IL10* production have a hereditary component estimated in 75%,[7] mainly due to the effect of promoter polymorphisms,[8] such as the three proximal single nucleotide polymorphisms (SNPs), at positions −1082 (A/G), −819(C/T), and −592(C/A) base pairs from the transcription start site.[8,9] Furthermore, genetic modifiers upstream of the transcription initiation site have been described.[10,11] However, the model of the genetic control of *IL10* expression has not reached a consensus yet, and results may vary according to cell type and *in vitro* activation stimuli.[12–14]

Due to its functions in B cell biology and its ability to induce a suppressor microenvironment, IL10 is an ongoing target in B-cell lymphoma research.[15,16] Classical Hodgkin lymphoma (cHL) is a B-cell neoplasm characterized by the presence of scarce tumor (Hodgkin-Reed-Sternberg, H-RS) cells surrounded by inflammatory non-neoplastic cells, collectively known as tumor microenvironment (TME), which pathogenic role is increasingly recognized.[17,18] In cHL adult patients, high IL10 serum levels are mainly associated with tumor burden (advanced disease stage, elevated LDH and β2-microglobulin levels) and unfavorable host-tumor factors (presence of B symptoms, anemia, low serum albumin levels), as well as a short survival.[19–24] Associations of *IL10* promoter polymorphisms with clinical outcome have been described in some studies with adult cHL patients,[25,26] while in others this association has not been found.[27,28] Moreover, an analysis of the

CONTACT Rocio Hassan chassan@inca.gov.br Oncovirology Laboratory, Bone Marrow Transplantation Center (CEMO), Instituto Nacional de Câncer – INCA, Praça Cruz Vermelha 23, 6° Andar, 20230-130, Rio de Janeiro, RJ Brazil.
Supplemental data for this article can be accessed on the publisher's website.
© 2018 Gabriela Vera-Lozada, Carolina Minnicelli, Priscilla Segges, Gustavo Stefanoff, Flavia Kristcevic, Joaquin Ezpeleta, Elizabeth Tapia, Gerald Niedobitek, Mário Henrique M. Barros, and Rocio Hassan. Published with license by Taylor & Francis Group, LLC
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License (http://creativecommons.org/licenses/by-nc-nd/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited, and is not altered, transformed, or built upon in any way.

relationship between *IL10* genotype and its influence in local mRNA expression and cellular profiles in the TME of cHL is lacking. The main goal of this study was to find clinical and biological correlates of *IL10* promoter proximal polymorphisms in children and adolescent with cHL. Given the important functions of this cytokine in the immune response, and the recognized role of TME in cHL therapeutic response, we hypothesize that clinical outcome imparted by *IL10* genetic variants may be mediated by effects on the tumor microenvironment composition or modulation.

Results

Clinical and epidemiological characteristics of patients

The clinical and histological characteristics of cHL patients have been described previously.[29] Median age at diagnosis was 14 years (3–18), and sex ratio (male:female) 1.8:1. Most patients presented with stages I/II and low-risk disease (62.1% and 52.6%, respectively), mediastinal mass was observed in 65.6% and extranodal disease in 11.6% of cases. Nodular sclerosis (NS) was the most frequently observed histological subtype (69%), followed by mixed cellularity (23%). Epstein-Barr virus (EBV) was detected in 44.8% of cases (Table S1).

IL10 polymorphisms

In total, 98 patients were successfully genotyped for *IL10* promoter rs1800896 (−1082A>G) and 97 for the rs1800872 (−592C>A) SNPs. Both SNPs were in Hardy-Weinberg equilibrium (−1082 $P = 0.53$ and −592 $P = 0.08$, Goodness of fit χ^2 test). Genotypic frequencies are described in Table 1. In view of the complete linkage of −819C/T and −592C/A SNPs,[30] the proximal haplotypes including the three positions (−1082A/G, −819C/T, and −592C/A) GCC, ACC and ATA were reconstructed in 96 patients. The frequency of haplotypes in cHL patients was 63.5% (61/96) for GCC; 46.9% (45/96) for ACC and 50% (48/96) for ATA.

IL10 gene expression

Levels of *IL10* mRNA in cHL lymph nodes [mean $2^{-\Delta Cq} \pm -2.515 \pm 1.531$ SD] were higher than the observed in reactive follicular hyperplasia (RFH) lymph nodes (mean $2^{-\Delta Cq}$: -3.757 ± 1.235 SD) ($P = 0.001$; Student's t test) (Fig. 1A).

In the cHL group, −1082GG genotype was associated with lower *IL10* mRNA expression (mean $2^{-\Delta Cq}$ -3.517 ± 2.009 SD) than AG+AA genotypes ($2^{-\Delta Cq}$: -2.346 ± 1.392) ($P = 0.014$; Student's t-test) (Fig. 1B). Genotypes of the −592 SNP showed no association with *IL10* mRNA expression (Fig. 1C). In the RFH group, no significant associations between −1082 or −592 SNP genotypes and *IL10* gene expression levels could be disclosed, likely due to the small number of samples analyzed. In respect of *IL10* haplotypes, a trend to high *IL10* expression in ATA and ACC carriers was observed, while the opposite occurred with GCC/GCC cHL cases (Fig. 1D).

IL10 genetic polymorphisms and mRNA expression in respect of clinical characteristics and therapy response

Patients with high *IL10* mRNA expression ($2^{-\Delta Cq} > -2.243$, median of the group) presented more frequently with B symptoms (64.3% *vs.* 35.7% in low expression patients; $P = 0.013$; χ^2 test). No associations between *IL10* promoter

Table 1. Progression-free survival (PFS) analysis according to genotypes and haplotypes in the *IL10* promoter in children and adolescent with classical Hodgkin lymphoma diagnosis.

			CI (95%) of univar.				CI (95%) of multivar.		
Variable	Number of Events	HR (Expβ) univar.	Lower	Upper	Univar. *P*-value	HR (Expβ) multivar.	Lower	Upper	Multivar. *P*-value
−1082*IL10*									
GG	0/16				$P = 0.065$[§]				
AG	10/39	0.495	0.239	0.939	$P = 0.031$	0.322	0.095	0.805	$P = 0.013$
AA	10/33								
GG	0/16				$P = 0.024$[§]				
AG+AA	20/72	0.095	0.001	0.691	$P = 0.013$	0.054	0.000	0.511	$P = 0.005$
−592*IL10*									
AA	5/10				$P = 0.009$[§]				
AC	8/31	2.350	1.278	4.291	$P = 0.007$	2.362	1.157	4.860	$P = 0.019$
CC	6/46								
CC	6/46				$P = 0.032$[§]				
AC+AA	13/41	0.378	0.138	0.932	$P = 0.034$	0.328	0.109	0.909	$P = 0.032$
Haplotypes									
ACC	9/43				$P = 0.721$[§]				
ATA+ GCC	10/43	0.853	0.347	2.068	$P = 0.723$	1.505	0.521	4.700	$P = 0.454$
ATA	13/41				$P = 0.035$[§]				
GCC+ ACC	6/45	2.601	1.055	7.119	$P = 0.038$	2.904	1.043	8.759	$P = 0.041$
GCC	10/55				$P = 0.204$[§]				
ACC+ATA	9/31	0.560	0.231	1.375	$P = 0.200$	0.405	0.102	1.361	$P = 0.454$

[§] *P*-values obtained by log-rank test. Other P-values calculated by Cox regression with Firth's correction strategies. In multivariate analysis was considered the follow variables: number of extranodal sites, high number of Granzyme B cells (median >25% of cells number in the tumoral microenvironment), leukopenia presence and mixed cellularity histological subtype. CI: confidence interval; Univar, univariate; Multivar, multivariate.

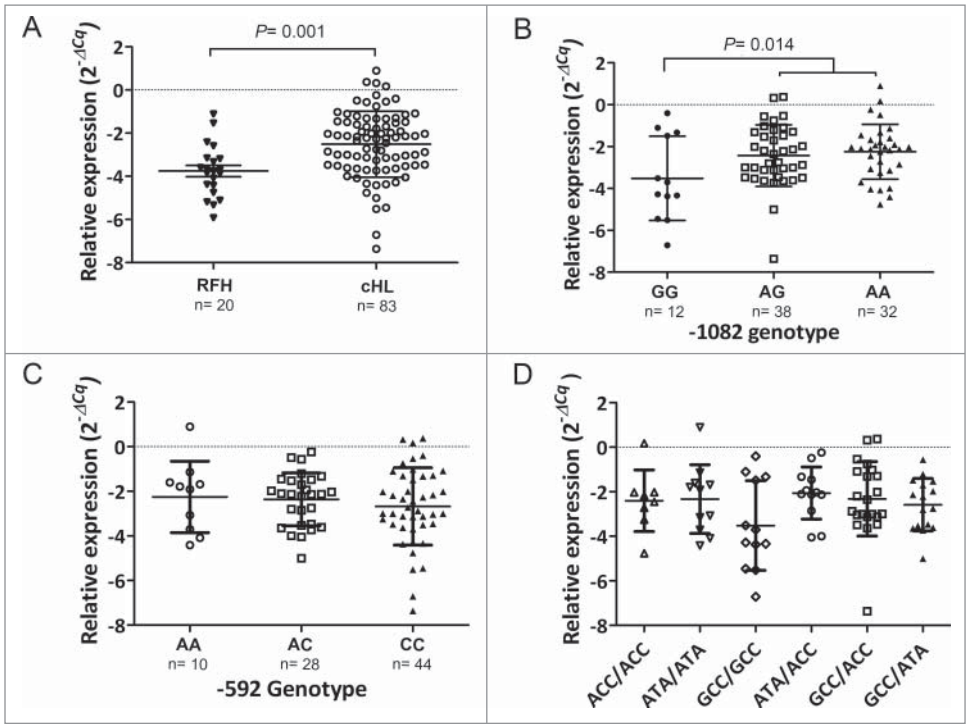

Figure 1. *IL10* gene expression. (A) *IL10* relative expression in classical Hodgkin lymphoma (cHL, n = 83) and reactive follicular hyperplasia (RFH, n = 20); (B) *IL10* relative expression in classical Hodgkin lymphoma lymph nodes according to *IL10* −1082A>G genotypes (AA, n = 32; AG, n = 38; GG, n = 12); (C) *IL10* relative expression in classical Hodgkin lymphoma lymph nodes according to *IL10* −592C>A genotypes (CC, n = 44; AC, n = 28; AA, n = 10); (D) *IL10* relative expression in classical Hodgkin lymphoma lymph nodes according to *IL10* haplotype (GCC/GCC, n = 12; ACC/ACC, n = 8; ATA/ATA, n = 11). $P < 0.05$ significant statistical association (Student's t-test).

polymorphisms or expression level were observed in respect of Ann-Arbor stage, age, histopathology subtypes, EBV status, extranodal commitment, and mediastinal mass.

Median follow up of the patient group was 65.5 months (72 months for censored patients). Progression-free survival (PFS) with 5 years follow-up was 78.6%. A poor PFS was asso-

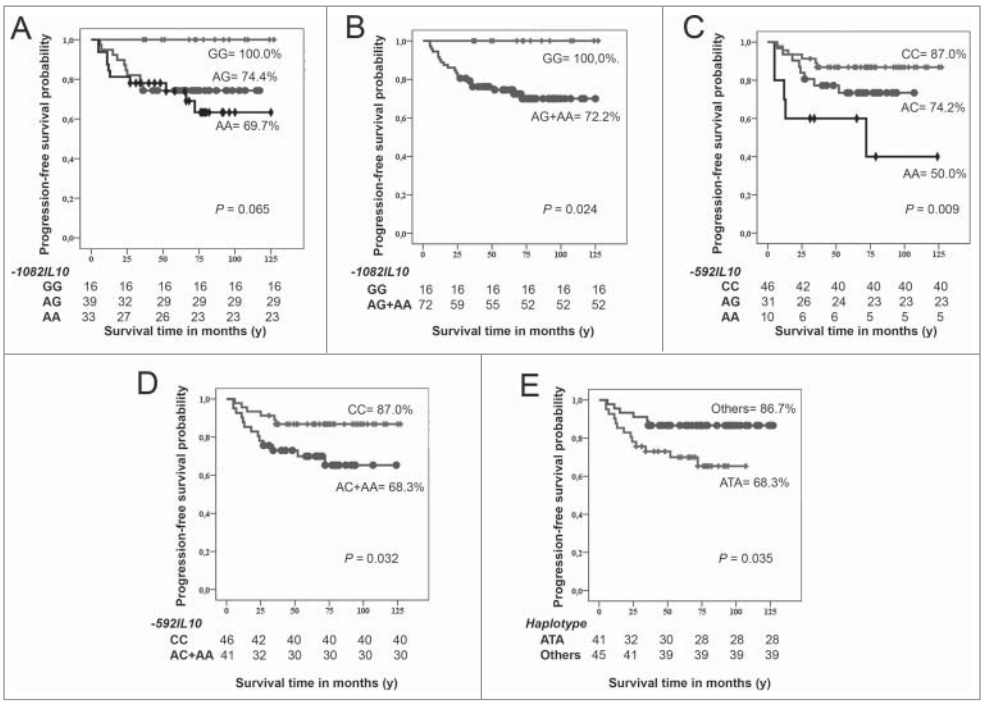

Figure 2. Kaplan-Meier curves for progression-free survival (PFS) of pediatric classical Hodgkin lymphoma according to evaluated IL10 promoter polymorphisms. (A) PFS according to *IL10* −1082A>G genotypes; (B) PFS comparing −1082GG *vs.* AG+AA genotype carriers; (C) PFS according to *IL10* −592C>A genotypes; (D) PFS comparing −592CC *vs.* AC+AA genotype carriers; (E) PFS of ATA haplotype carriers *vs.* others haplotypes. $P < 0.05$ significant statistical association (log-rank test).

ciated, in univariate analysis, with leukopenia, extranodal disease, MC subtype and high numbers of Granzyme B+ lymphocytes, as described previously.[29,31]

Genotypes of the −592A/C SNP influenced prognosis, with worse PFS exhibited by −592AA patients (50%), when compared with AC (74.2%), and CC patients (87.0%) ($P = 0.009$, log-rank test). Patients with the −1082GG genotype showed a better PFS (100%) than AG and AA carriers (72.2%) ($P = 0.024$, log-rank test) (Table 1 and Figs. 2A-D).

Patients carrying the ATA haplotype showed worse PFS (68.3%) when compared to other haplotypes (86.7%) ($P = 0.035$, log-rank test; Fig. 2E). The significance was maintained after application of Firth'correction (Table 1). Thus, genotypes and haplotypes associated with high *IL10* expression levels were shown to be associated to a shorter PFS. None of the *IL10* genetic variants were associated with overall survival (OS) (Table S2).

In multivariate analysis performed by Firth's penalized Cox regression, considering the clinical and microenvironment variables with described PFS impact, −10822 GG genotype (HR: 0.054, 95% CI 0.000–0.511, $P = 0.005$), the −592 CC genotype (HR: 0.328, 95% CI 0.109–0.909, $P = 0.032$) and the ATA haplotype (HR: 2.904, 95% CI 1.043–8.759; $P = 0.041$) retained prognostic impact (Table 2).

Table 2. Multivariate Cox regression with Firth correction, considering *IL10* genotypes/haplotypes along with other clinical and microenvironment variables influencing PFS in pediatric classical Hodgkin lymphoma. (A) Model I, −1082 GG genotype; (B) Model II, –592 CC genotype; (C) Model III, ATA haplotype.

(A)

		Confidence Interval (95%)		
Variable	HR (Expβ)	Lower	Upper	*P*-value
Extranodal sites	6.641	1.820	21.684	0.006
High number of Granzyme B cells	4.179	1.218	22.122	0.021
Leukopenia	2.822	0.848	7.756	0.086
Mixed cellularity	3.637	1.242	10.076	0.020
−10822 GG genotype	0.054	0.000	0.511	0.005

This multivariate analysis was performed with 74 patients.

(B)

		Confidence Interval (95%)		
Variable	HR (Expβ)	Lower	Upper	*P*-value
Extranodal sites	4.451	1.314	12.716	0.019
High number of Granzyme B cells	6.209	1.504	57.200	0.008
Leukopenia	3.313	0.823	10.535	0.086
Mixed cellularity	2.850	0.978	7.753	0.055
−592 CC genotype	0.328	0.109	0.909	0.032

This multivariate analysis was performed with 73 patients.

(C)

		Confidence Interval (95%)		
Variable	HR (Expβ)	Lower	Upper	*P*-value
Extranodal sites	4.265	1.260	12.174	0.022
High number of Granzyme B cells	6.853	1.622	63.888	0.006
Leukopenia	3.265	0.811	10.402	0.089
Mixed cellularity	2.926	1.007	7.923	0.049
ATA haplotype	2.904	1.043	8.759	0.041

This multivariate analysis was performed with 72 cHL patients.

Association of IL10 genetic variants with the tumor microenvironment cell composition

In view that genotypes and haplotypes determining high levels of *IL10* mRNA in lymph nodes were associated to an unfavorable prognosis, we next asked if *IL10* genetic background may dictate some aspects of the TME composition in cHL. In this work, given the roles of MAF transcription factor in the control of *IL10* expression,[32,33] expression of MAF by the TME cells was used as a surrogate for cell commitment to express *IL10*.

Distribution of lymphocyte and macrophage sub-populations in the TME in relation to age group, histology, EBV-status and their prognostic impact have been previously reported.[29,31] MAF expression by H-RS cells was observed in only two cases and in few cells. Patients with −1082GG (low *IL10* expression) and −592CC genotypes exhibited low numbers of MAF+ inflammatory cells (median: 20 *vs.* 78 cells/mm^2, and median: 49 *vs.* 108 cells/mm^2; $P = 0.012$ and $P = 0.003$, respectively; Mann-Whitney test) (Fig. 3A, B). Moreover, −1082GG patients exhibited low percentages of CD68+MAF+ macrophages (15.04% *vs.* 47.26% for the other genotypes, $P = 0.017$; Mann-Whitney test) (Fig. 3C, Table S3). Conversely, ATA haplotype (high level *IL10* expression) was associated with high numbers of MAF+ inflammatory cells (median 108 *vs.* 49 cells/mm^2, $P = 0.005$; Mann-Whitney test) (Fig. 3D, Table S3).

Since EBV may modulate the TME, with EBV-associated cases being characterized by significantly higher numbers of cytotoxic/Th1 lymphocytes and macrophages than EBV-group[29,31] we decided to investigate potential interactions between EBV and the studied SNPs in the TME composition. For this, we have defined a ratio of FOXP3+ over CD8+ and TBET+ cells; and MAF+ over CD8+ and TBET+ cells as indicating a predominantly Th2/regulatory TME, and then analyzed these cell population balances in cases stratified by *IL10* genotypes and EBV status.

Analyses were conducted by linear logistic regression using log10-transformed raw cell ratios as dependent variable. The MAF+/TBET+ ratio showed to be inversely dependent on both, the EBV presence and low *IL10* expression-associated −1082GG genotype ($P = 0.020$), or −592CC genotype ($P = 0.031$), or GCC haplotype ($P = 0.045$) (Table S4), indicating a significant effect of both EBV and *IL10* genotypes/haplotypes in the TME modulation (Fig. 4).

Discussion

A large number of studies have demonstrated that H-RS cells may be able to modulate their microenvironment, e.g., by the production of cytokines and chemokines, contributing to an immunosuppressive TME and survival of these neoplastic cells.[18,34,35] In this context, *IL10* functional genetic variants that are being strongly considered in the search for prognostic markers in cHL, may also be factors of the disease histopathogenesis.

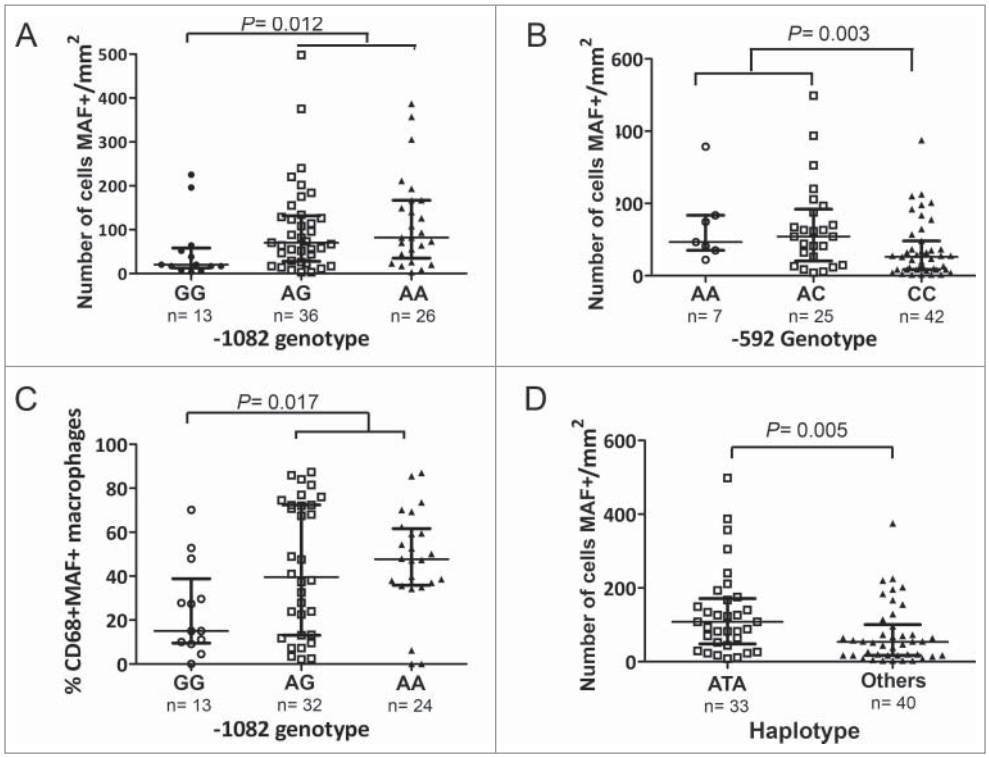

Figure 3. Number of cells expressing the MAF transcription factor according to *IL10* genotypes and haplotypes. (A) Numbers of MAF+ cells according to −1082 genotypes (AA, n = 26; AG, n = 36; GG, n = 13); (B) Numbers of MAF+ cells according to −592C>A genotypes (CC, n = 42; AC, n = 25; AA, n = 7); (C) Percentages of CD68+MAF+ macrophages according to −1082A>G genotypes (AA, n = 24; AG, n = 32; GG, n = 13); (D) Numbers of MAF+ cells in ATA haplotype (n = 33) *vs.* carriers of other haplotypes (n = 40). $P < 0.05$ significant statistical association (Mann-Whitney test).

Our first aim was to validate the impact of *IL10* polymorphisms on disease prognosis in our series of pediatric cHL. In our pediatric cohort, the −1082AA+AG genotypes and ATA haplotype were associated with unfavourable prognosis, in agreement with previous results in adult cHL patients, in which an unfavourable outcome was associated with *IL10* ATA/ATA haplotypes[26] and the presence of the −592AA genotype.[25] Considering ours as an independent validation cohort, our results give support to the clinical importance of *IL10* genetic variants in the full spectrum of cHL, by demonstrating an association also in the pediatric population.

We next intended to find phenotypic correlates that may help to explain ours and other's clinical results. Our methodological approach to draw genotype-phenotype associations was

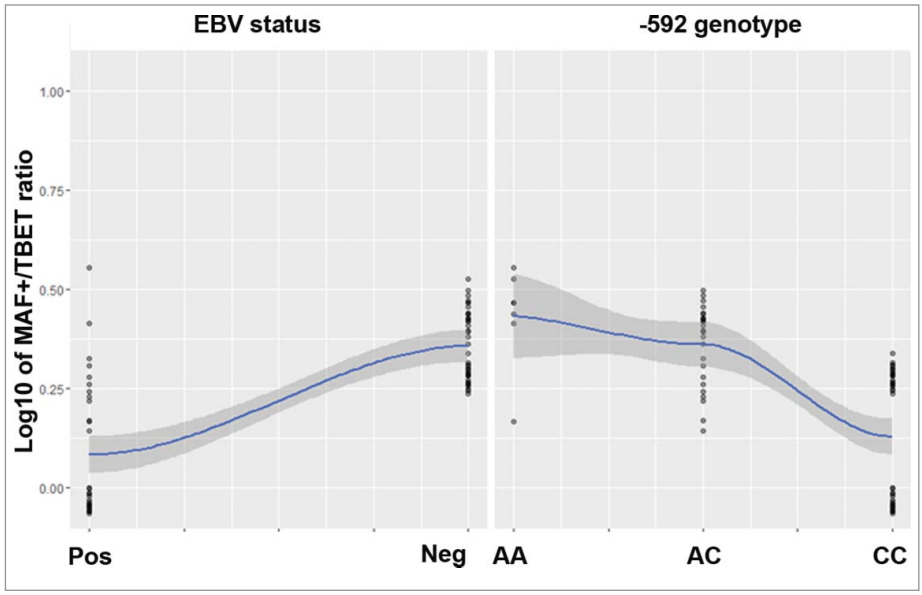

Figure 4. Main effect plots of EBV status and −592 genotypes on the tumor microenvironment polarization, measured by the distribution of the MAF+/TBET+ cell ratios. Variables are normalized to a 0–1 range. The graphic was constructed with the statistical R environment.

based on the quantification of mRNA levels and cells in tumor tissues, the higher levels of *IL10* expression in cHL lymph nodes *vs.* RFH lymph nodes pointed to the immunosupressor phenotype as a pathogenic factor in the former condition.

In studies based on IL10 systemic levels, it is still a matter of debate whether IL10 levels reflect its direct participation in cHL pathophysiology, or merely reflect the effect of tumor burden on a drained immune system. Our findings of high levels of tumor *IL10* expression associated with B symptoms, as well as the association of high-expression *IL10* promoter polymorphisms with an unfavorable therapeutic response reinforce the idea of a direct role of IL10 in cHL pathogenesis and are in line with several studies reporting high IL10 serum levels associated to unfavorable disease characteristics, therapy response and short survival in cHL patients.[19–24,36]

We next addressed the phenotypic correlations of *IL10* proximal promoter polymorphisms. In our system, which relies not in experimental cell activation, but in the state of activation and number of infiltrating and H-RS cells in tumor lymph nodes, we found that −1082A/G genotypes have a leading role in modulating *IL10* expression, with ATA-associated genotypes and haplotypes contributing to *IL10* high expression levels. The association of −1082GG genotype with low expression was somewhat surprising, since previous studies have found this genotype associated with IL10 high expression levels.[9,13] However, *in vitro* assays have shown that the −1082A allele was associated with the highest IL10 production when the position was isolated against a constant haplotype background.[37] The −1082A allele was also correlated with high IL10 expression in whole blood from rheumatoid arthritis patients stimulated in vitro with lipopolysaccharide;[38] in peripheral blood mononuclear cells stimulated with ConA;[39] as well as in plasma of healthy individuals.[40]

Discrepancy with the studies that found −1082GG genotypes associated with high expression levels[9,13] may be a consequence of different activation conditions and of the diversity in cell compositions of the experimental models. In fact, it has been described, but not yet completely explored in complex systems, that *IL10* promoter occupancy may vary according to cell lineages (i.e. lymphocytes, monocytes and macrophages)[3] where epigenetic mechanisms might be modulated by the diverse microenvironment stimuli.

In agreement with that concept, we observed scarce numbers of MAF+ neoplastic cells, while MAF expression by inflammatory cells was variable, pointing to diverse IL10 activating pathways in the different cell lineages.[3,32,41]

IL10 genetic variants determining high *IL10* mRNA levels were furthermore associated with high numbers of inflammatory cells expressing MAF. Thus, assuming the premise that MAF is an important *IL10* transcription factor in immune cells,[32] the model of *IL10* mRNA genetic control was replicated at the cellular level, at least in the TME. Since the main associations were observed between *IL10* polymorphisms and MAF expression by inflammatory cells, the action in *cis* of *IL10* polymorphisms on the MAF ligation domain in *IL10* promoter is a probable explanation. The MAF recognition element (MARE) localizes to −196/−184 in the *IL10* promoter, and has been demonstrated functional by both *in vitro* (EMSA) and *in vivo* (ChIP assay) experiments.[32,33] It is likely therefore that one of the effects of the proximal promoter polymorphisms is to modulate the binding of MAF to its recognition element in *IL10* promoter, thereby leading to a more elevated *IL10* expression rate. High expression levels of *IL10* in serum and TME might then mediate a positive loop of enrichment in monocytes with an immunosuppressive phenotype (and consequently M2-like macrophages) as described in B cell non-Hodgkin lymphoma.[42]

Additionally, we were able to disclose an inverse correlation of *IL10* expression levels with the number of TBET+ lymphocytes (Th1) and a subset of dendritic cells. This may reflect the inverse numerical relationships in Th1-oriented and Th2/Treg-oriented microenvironments[43,44] and allows hypotheses about a yet unproven role of the cytokine in the *in situ* differentiation and activation of intratumoral lymphocytes and dendritic cells.

Macrophages are plastic cells with potentiality to both pro-inflammatory and regulatory functions.[6] The role of tumor-associated macrophages in the prognosis of cHL is still controversial, with some studies showing association of high counts/density of TAM with poor survival in adult cHL,[45–47] while some others failed to disclose such association.[48,49] This discrepancy may be due to differences in immunohistochemical biomarkers and scoring systems.[50] However, it is possible that part of this lack of reproducibility results from TAMs heterogeneity. In fact, in pediatric cHL, we have recently shown that M2-like macrophages, and not M1 macrophages, were associated with a poor outcome.[31] Moreover, the pathogenic role of immunosuppressive macrophages in cHL is being highlighted in preclinical studies targeting TAMs with chimeric antigen receptor T cell (CART) therapy.[51]

In this work, we extended our previous results, by showing that percentages of intratumoral MAF-expressing, M2-like-polarized macrophages were correlated with *IL10* genotypes, suggesting a role of the host genetic background in the susceptibility to polarize intratumoral macrophages to a suppressor phenotype, indirectly participating in the microenvironment shaping.

In this study, we were not able to disclose any consistent association of EBV with *IL10* polymorphisms or MAF expression. Previous studies have shown an increased IL10 production in EBV+ H-RS cells[52,53] and a role of *IL10* expression by H-RS cells in the evasion of viral-directed cytotoxicity,[54] suggesting that IL10 might mediate local effects of autocrine stimuli and/or immune escape in H-RS cells. In this study, *IL10* expression level was measured by mRNA analysis of the whole lymph node, thus detected levels represent the contribution of both, H-RS and infiltrating cells. In that regard, since EBV in pediatric cHL is associated with a cytotoxic/Th1 oriented TME,[29,31] not being able to detect a difference regarding *IL10* expression level in EBV+ and EBV- cases is not surprising. Further studies to better discriminate the patterns of *IL10* expression at the cellular and molecular level by tumor *vs.* infiltrating cells in respect of EBV status would help to clarify this issue, including the interaction between EBV- and IL10-mediated TME modulations.

To our knowledge, this is a first study to show an association between *IL10* genotype and phenotype in patients

with cHL at the molecular, cellular, and clinical levels. While we are aware of the limitations imposed by the number of patients, we reinforce that the main goal of this study was drawing biological and immunological correlates from meaningful clinical factors, to help highlighting pathogenic mechanisms in a complex disease such as cHL. On the whole, our results contribute to fill a gap in the knowledge of the relationship between *IL10* genotype and phenotype in cancer, and advance the concept of genetic control of microenvironment composition as the basis of susceptibility and therapeutic response.

Materials and methods

Patients and samples

Ninety-eight HIV-negative children and adolescents (up to 18 year old) diagnosed with cHL at the Instituto Nacional de Câncer (INCA), Rio de Janeiro, Brazil, between 1999 and 2006, were included in this study. All included patients in this study were evaluated by a minimal follow-up of 60 months.

Diagnosis of cHL was based on morphologic criteria[55] and immunohistochemical (IHC) characterization.[56] Latent EBV infection has been investigated previously in all cHL cases by EBER-ISH hybridization.[56] All patients were treated according to adriamycin-based standard pediatric protocols, as described.[29] Additionally, 20 patients with HIV-negative RFH diagnosis were included as controls of *IL10* expression (median age: 36 year, 4 – 83). This study was approved by the INCA Ethics Committee (Number 37/05 and CAAE 56999916.5.0000.5274) and all patients were included after signed informed consent.

Nucleic acids extraction

DNA was extracted using QIAamp® DNA FFPE Tissue (Qiagen®, catalog number 56404) from three to five microtomized sections (3 μm) of formalin-fixed, paraffin-embedded (FFPE) lymph nodes. Total RNA was obtained with the Master Pure™ kit (Epicentre®, catalog number MCR85102), as described.[57] All working conditions were RNase-free. The quantity and purity of nucleic acids was evaluated using a Nanodrop®, ND-1000 Spectrophotometer (Wilmington, Delaware USA) at λ260/280/230 ratios, and additionally, a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) for RNA quality.

IL10 genotyping

The single nucleotide polymorphisms (SNPs) rs1800896 (−1082A>G) and rs1800872 (−592C>A) (catalog number: C_1747360_10 and C_1747363_10, respectively) were genotyped using TaqMan® assays in a Viia7 platform (Applied Biosystems, Life Technologies™, Carlsbad, CA), using 3 ng/μL of DNA in 15 μL final volume. Thermal profile was 50°C for 2 min, 95°C for 10 min and 50 cycles at 92°C for 15 s and at 60°C for 90 s. A post-read step of 1 min at 60°C allowed allelic discrimination. Controls with known *IL10* genotypes (2 samples for each homozygote and heterozygote genotype) as well as 2 negative template controls were included in each run; 10% of samples were randomly selected to be re-genotyped in the next run.

IL10 gene expression

cDNA was prepared from 500 ng of total RNA in 20 μL final volume, using High-Capacity cDNA Archive kit (Applied Biosystems, catalog number 4368814). A pre-amplification step was performed, using the TaqMan® PreAmp Master Mix (Applied Biosystems). *IL10* expression was quantified using a TaqMan® assays (Hs00961622_m1, Applied Biosystems) in 15 μL final volume with standard 50-cycles thermal cycling. *GUSB* (Hs99999908_m1) and *HMBS* (Hs00609297_m1) were used as reference genes. Each measurement was performed in duplicate and quantified by *Cq*-value with fixed thresholds. Samples were considered amplifiable with *Cq*-values <35 cycles, and only duplicates with SD ≤0.15 cycles were accepted. The quantification values were expressed as log2 (2-ΔCq) after normalization with the mean level expression of the reference genes.[58]

Immunohistochemical characterization of tumor microenvironment

A tissue microarray (TMA) block was built as described previously.[29] The immune cells from the TME were identified by single or double immunohistochemistry, as described previously,[29,31,56,59] with primary antibodies described in Table S5. Briefly, immunodetection was performed using ZytoChem Plus HRP polymer kit (Zytomed Systems, catalog number: POLHRP-100), employing diaminobenzidine (DAB) chromogen as substrate for single IHC techniques, and with AP Polymer System (Zytomed Systems, catalog number: POLAP-100), employing Blue Alkaline Phosphatase (Vector Laboratories, catalog number: SK-5300) as substrate for double detection IHC. IL10 producing cells were identified by the expression of MAF, an essential transcription factor for *IL10* gene expression in T lymphocytes and macrophages.[3,32,60] The computer assisted cell quantification was performed with the image analysis software HISTO (Biomas, Erlangen, Germany), as described previously.[29]

The identification of all MAF+ cells were performed by single IHC, while the specific identification of MAF+ macrophages was performed by double IHC.[59] MAF+ inflammatory cells and MAF+ macrophages were expressed as absolute number/mm^2. Additionally, MAF+ macrophages were expressed as a percent value of CD68+ or CD163+ macrophages, as follow (number of MAF-expressing CD68+ or CD163+ macrophages/ total number of CD68+ or CD163+ macrophages) x 100.

Statistical analyses

Student's t test and one-way ANOVA were used for comparing gene expression levels of two or multiple groups. Mann-Whitney's test was used to analyze associations between dichotomous and continuous non normal variables such as cell numbers in the TME, while Spearman's test was used for correlating

continuous variables. Pearson's chi-square and Fisher's exact test were used for testing association in dichotomous variables. *P*-values <0.05 were considered as significant in 2-tailed tests. PFS was the time in months between diagnoses to relapse associated to cHL, initiation of other unplanned treatment or last follow-up, and OS the interval in months between diagnosis to death by any cause or last follow-up. Kaplan-Meier method and the log-rank test were used for estimating and comparing the distribution of survival probabilities. Additionally, univariate and multivariate penalized Firth logistic regressions were performed to reduce possible bias estimation effects due to small sample number. The proportionality assumptions for multivariate analysis were analyzed by time dependent covariance ($P > 0.05$ assumption satisfied). Higher order interactions between SNP and clinical parameters were not investigated. Statistical Package for the Social Sciences (SPSS) 20.0 software and CRAN R-project were used for statistical analyses.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We are indebted to Dr. David Enot (Institut de Cancérologie Gustave Roussy, Villejuif) and an anonymous reviewer for the useful suggestions that helped to improve this manuscript. This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) Grant 475969/2013-8 and INCT para Controle do Câncer, Brazil (Grants CNPq 573806/2008-0 and FAPERJ E26/170.026/2008). Dr. Mário Barros was supported by Alexander von Humboldt Foundation. The authors declare no potential conflicts of interest.

Authorship

Contribution: G.V.L. designed and performed the experiments, collected and analyzed data and wrote the manuscript; CM performed genotyping experiments, collected data and reviewed the manuscript; P.S., performed TME characterization and reviewed the manuscript C.G.S. recruited cases, analyzed data and reviewed the manuscript; F.K., J.E. and E.T. performed statistical analysis, analyzed data and reviewed the manuscript; G.N. reviewed the cases, supervised TME characterization and reviewed the manuscript; M.H.M.B. reviewed cases, performed and supervised TME characterization, and wrote the manuscript; R.H. designed and supervised the experiments, analyzed data and wrote the manuscript.

ORCID

Gabriela Vera-Lozada http://orcid.org/0000-0003-4398-337X
Rocio Hassan http://orcid.org/0000-0003-3782-3580

References

1. Iyer SS, Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. Crit Rev Immunol. 2012;32:23–63. doi:10.1615/CritRevImmunol.v32.i1.30. PMID:22428854.
2. Burdin N, Rousset F, Banchereau J. B-cell-derived IL-10: production and function. Methods San Diego Calif. 1997;11:98–111. doi:10.1006/meth.1996.0393.
3. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. Nat Rev Immunol. 2010;10:170–81. doi:10.1038/nri2711. PMID:20154735.
4. Ng THS, Britton GJ, Hill EV, Verhagen J, Burton BR, Wraith DC. Regulation of Adaptive Immunity; The Role of Interleukin-10. Front Immunol [Internet] 2013 [cited 2014 Mar 24]; 4. Available from: http://www.frontiersin.org/Journal/10.3389/fimmu.2013.00129/full. doi:10.3389/fimmu.2013.00129.
5. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. J Immunol Baltim Md 1950. 2008;180:5771–7.
6. Biswas SK, Allavena P, Mantovani A. Tumor-associated macrophages: functional diversity, clinical significance, and open questions. Semin Immunopathol. 2013;35:585–600. doi:10.1007/s00281-013-0367-7. PMID:23657835.
7. Reuss E, Fimmers R, Kruger A, Becker C, Rittner C, Höhler T. Differential regulation of interleukin-10 production by genetic and environmental factors–a twin study. Genes Immun. 2002;3:407–13. doi:10.1038/sj.gene.6363920. PMID:12424622.
8. Eskdale J, Gallagher G, Verweij CL, Keijsers V, Westendorp RG, Huizinga TW. Interleukin 10 secretion in relation to human IL-10 locus haplotypes. Proc Natl Acad Sci U S A. 1998;95:9465–70. doi:10.1073/pnas.95.16.9465. PMID:9689103.
9. Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ, Hutchinson IV. An investigation of polymorphism in the interleukin-10 gene promoter. Eur J Immunogenetics Off J Br Soc Histocompat Immunogenetics. 1997;24:1–8. doi:10.1111/j.1365-2370.1997.tb00001.x.
10. Kube D, Rieth H, Eskdale J, Kremsner PG, Gallagher G. Structural characterisation of the distal 5′ flanking region of the human interleukin-10 gene. Genes Immun. 2001;2:181–90. doi:10.1038/sj.gene.6363750. PMID:11477472.
11. Rieth H, Mörmann M, Luty AJF, Assohou-Luty CA, Roupelieva M, Kremsner PG, Kube D. A three base pair gene variation within the distal 5′-flanking region of the interleukin-10 (IL-10) gene is related to the in vitro IL-10 production capacity of lipopolysaccharide-stimulated peripheral blood mononuclear cells. Eur Cytokine Netw. 2004;15:153–8. PMID:15319176.
12. Kurreeman FAS. Transcription of the IL10 gene reveals allele-specific regulation at the mRNA level. Hum Mol Genet. 2004;13:1755–62. doi:10.1093/hmg/ddh187. PMID:15198994.
13. Mörmann M, Rieth H, Hua TD, Assohou C, Roupelieva M, Hu SL, Kremsner PG, Luty AJF, Kube D. Mosaics of gene variations in the Interleukin-10 gene promoter affect interleukin-10 production depending on the stimulation used. Genes Immun. 2004;5:246–55. doi:10.1038/sj.gene.6364073. PMID:15116066.
14. Larsson L, Rymo L, Berglundh T. Sp1 binds to the G allele of the- 1087 polymorphism in the IL-10 promoter and promotes IL-10 mRNA transcription and protein production. Genes Immun. 2010;11:181–7. doi:10.1038/gene.2009.103. PMID:20072143.
15. Khatri VP, Caligiuri MA. A review of the association between interleukin-10 and human B-cell malignancies. Cancer Immunol Immunother CII. 1998;46:239–44. doi:10.1007/s002620050483. PMID:9690451.
16. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001;19:683–765. doi:10.1146/annurev.immunol.19.1.683. PMID:11244051.
17. Küppers R. The biology of Hodgkin's lymphoma. Nat Rev Cancer. 2009;9:15–27. doi:10.1038/nrc2542. PMID:19078975.
18. Steidl C, Connors JM, Gascoyne RD. Molecular pathogenesis of Hodgkin's lymphoma: increasing evidence of the importance of the microenvironment. J Clin Oncol Off J Am Soc Clin Oncol. 2011;29:1812–26. doi:10.1200/JCO.2010.32.8401.
19. Blay JY, Burdin N, Rousset F, Lenoir G, Biron P, Philip T, Banchereau J, Favrot MC. Serum interleukin-10 in non-Hodgkin's lymphoma: a prognostic factor. Blood. 1993;82:2169–74. PMID:8400266.
20. Bohlen H, Kessler M, Sextro M, Diehl V, Tesch H. Poor clinical outcome of patients with Hodgkin's disease and elevated interleukin-10 serum levels. Clinical significance of interleukin-10 serum levels for Hodgkin's disease. Ann Hematol. 2000;79:110–3. doi:10.1007/s002770050564. PMID:10803931.
21. Viviani S, Notti P, Bonfante V, Verderio P, Valagussa P, Bonadonna G. Elevated pretreatment serum levels of Il-10 are associated with a poor prognosis in Hodgkin's disease, the milan cancer institute

experience. Med Oncol Northwood Lond Engl. 2000;17:59–63. doi:10.1007/BF02826218.
22. Vassilakopoulos TP, Nadali G, Angelopoulou MK, Siakantaris MP, Dimopoulou MN, Kontopidou FN, Rassidakis GZ, Doussis-Anagnostopoulou IA, Hatzioannou M, Vaiopoulos G, et al. Serum interleukin-10 levels are an independent prognostic factor for patients with Hodgkin's lymphoma. Haematologica. 2001;86:274–81. PMID:11255274.
23. Rautert R, Schinköthe T, Franklin J, Weihrauch M, Boll B, Pogge E, Bredenfeld H, Engert A, Diehl V, Re D. Elevated pretreatment interleukin-10 serum level is an International Prognostic Score (IPS)-independent risk factor for early treatment failure in advanced stage Hodgkin lymphoma. Leuk Lymphoma. 2008;49:2091–8. doi:10.1080/10428190802441339. PMID:19021051.
24. Hohaus S, Giachelia M, Massini G, Vannata B, Criscuolo M, Martini M, D'Alo' F, Voso MT, Larocca LM, Leone G. Clinical significance of interleukin-10 gene polymorphisms and plasma levels in Hodgkin lymphoma. Leuk Res. 2009;33:1352–6. doi:10.1016/j.leukres.2009.01.009. PMID:19201467.
25. Hohaus S, Giachelia M, Di Febo A, Martini M, Massini G, Vannata B, D'Alo' F, Guidi F, Greco M, Pierconti F, et al. Polymorphism in cytokine genes as prognostic markers in Hodgkin's lymphoma. Ann Oncol. 2007;18:1376–81. doi:10.1093/annonc/mdm132. PMID:17496310.
26. Schoof N, Franklin J, Furst R, Zander T, von Bonin F, Peyrade F, Trumper L, Diehl V, Engert A, Kube D, et al. Interleukin-10 Gene Polymorphisms are Associated With Freedom From Treatment Failure for Patients With Hodgkin Lymphoma. The Oncologist. 2013;18:80–9. doi:10.1634/theoncologist.2012-0291. PMID:23299779.
27. Yri OE, Ekstrøm PO, Hilden V, Gaudernack G, Liestøl K, Smeland EB, Holte H. Polymorphisms in genes encoding interleukin-10 and drug metabolizing enzymes GSTP1, GSTT1, GSTA1 and UGT1A1 influence risk and outcome in Hodgkin lymphoma. Leuk Lymphoma. 2012;53:1934–44. doi:10.3109/10428194.2012.682307. PMID:22475179.
28. Ghesquières H, Maurer MJ, Casasnovas O, Ansell SM, Larrabee BR, Lech-Maranda E, Novak AJ, Borrel A-L, Slager SL, Brice P, et al. Cytokine gene polymorphisms and progression-free survival in classical Hodgkin lymphoma by EBV status: results from two independent cohorts. Cytokine. 2013;64:523–31. doi:10.1016/j.cyto.2013.08.002. PMID:24008079.
29. Barros MHM, Vera-Lozada G, Soares FA, Niedobitek G, Hassan R. Tumor microenvironment composition in pediatric classical Hodgkin lymphoma is modulated by age and Epstein-Barr virus infection. Int J Cancer. 2012;131:1142–52. doi:10.1002/ijc.27314. PMID:22025264.
30. Eskdale J, Kube D, Gallagher G. A second polymorphic dinucleotide repeat in the 5′ flanking region of the human IL10 gene. Immunogenetics. 1996;45:82–3. doi:10.1007/s002510050174. PMID:8881045.
31. Barros MHM, Segges P, Vera-Lozada G, Hassan R, Niedobitek G. Macrophage Polarization Reflects T Cell Composition of Tumor Microenvironment in Pediatric Classical Hodgkin Lymphoma and Has Impact on Survival. PLOS ONE. 2015;10:e0124531. doi:10.1371/journal.pone.0124531. PMID:25978381.
32. Cao S, Liu J, Song L, Ma X. The protooncogene c-Maf is an essential transcription factor for IL-10 gene expression in macrophages. J Immunol. 2005;174:3484–92. doi:10.4049/jimmunol.174.6.3484. PMID:15749884.
33. Xu J, Yang Y, Qiu G, Lal G, Wu Z, Levy DE, Ochando JC, Bromberg JS, Ding Y. c-Maf Regulates IL-10 Expression during Th17 Polarization. J Immunol. 2009;182:6226–36. doi:10.4049/jimmunol.0900123. PMID:19414776.
34. Skinnider BF, Mak TW. The role of cytokines in classical Hodgkin lymphoma. Blood. 2002;99:4283–97. doi:10.1182/blood-2002-01-0099. PMID:12036854.
35. Aldinucci D, Gloghini A, Pinto A, De Filippi R, Carbone A. The classical Hodgkin's lymphoma microenvironment and its role in promoting tumour growth and immune escape. J Pathol. 2010;221:248–63. doi:10.1002/path.2711. PMID:20527019.
36. Gaiolla RD, Domingues MAC, Niéro-Melo L, de Oliveira DE. Serum levels of interleukins 6, 10, and 13 before and after treatment of classic Hodgkin lymphoma. Arch Pathol Lab Med. 2011;135:483–9. PMID:21466366.
37. Eskdale J, Keijsers V, Huizinga T, Gallagher G. Microsatellite alleles and single nucleotide polymorphisms (SNP) combine to form four major haplotype families at the human interleukin-10 (IL-10) locus. Genes Immun. 1999;1:151–5. doi:10.1038/sj.gene.6363656. PMID:11196662.
38. Huizinga TW, Keijsers V, Yanni G, Hall M, Ramage W, Lanchbury J, Pitzalis C, Drossaers-Bakker WK, Westendorp RG, Breedveld FC, et al. Are differences in interleukin 10 production associated with joint damage? Rheumatology. 2000;39:1180–8. doi:10.1093/rheumatology/39.11.1180. PMID:11085795.
39. Warlé MC, Farhan A, Metselaar HJ, Hop WC, Perrey C, Zondervan PE, Kap M, Kwekkeboom J, Ijzermans JN, Tilanus HW, et al. Are cytokine gene polymorphisms related to in vitro cytokine production profiles? Liver Transpl. 2003;9:170–81. doi:10.1053/jlts.2002.50014. PMID:12548511.
40. Helminen ME, Kilpinen S, Virta M, Hurme M. Susceptibility to primary Epstein-Barr virus infection is associated with interleukin-10 gene promoter polymorphism. J Infect Dis. 2001;184:777–80. doi:10.1086/322987. PMID:11517440.
41. Yang Y, Ochando J, Yopp A, Bromberg JS, Ding Y. IL-6 plays a unique role in initiating c-Maf expression during early stage of CD4T cell activation. J Immunol Baltim Md 1950. 2005;174:2720–9.
42. Xiu B, Lin Y, Grote DM, Ziesmer SC, Gustafson MP, Maas ML, Zhang Z, Dietz AB, Porrata LF, Novak AJ, et al. IL-10 induces the development of immunosuppressive CD14(+)HLA-DR(low/−) monocytes in B-cell non-Hodgkin lymphoma. Blood Cancer J. 2015;5:e328. doi:10.1038/bcj.2015.56. PMID:26230952.
43. Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Altern Med Rev. 2003;8:223–46. PMID:12946237.
44. Skapenko A, Niedobitek GU, Kalden JR, Lipsky PE, Schulze-Koops H. Generation and regulation of human Th1-biased immune responses in vivo: a critical role for IL-4 and IL-10. J Immunol Baltim Md 1950. 2004;172:6427–34.
45. Steidl C, Lee T, Shah SP, Farinha P, Han G, Nayar T, Delaney A, Jones SJ, Iqbal J, Weisenburger DD. Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. N Engl J Med. 2010;362:875–85. doi:10.1056/NEJMoa0905680. PMID:20220182.
46. Tzankov A, Matter MS, Dirnhofer S. Refined Prognostic Role of CD68-Positive Tumor Macrophages in the Context of the Cellular Micromilieu of Classical Hodgkin Lymphoma. Pathobiology. 2010;77:301–8. doi:10.1159/000321567. PMID:21266828.
47. Kamper P, Bendix K, Hamilton-Dutoit S, Honore B, Nyengaard JR, d'Amore F. Tumor-infiltrating macrophages correlate with adverse prognosis and Epstein-Barr virus status in classical Hodgkin's lymphoma. Haematologica. 2011;96:269–76. doi:10.3324/haematol.2010.031542. PMID:21071500.
48. Azambuja D, Natkunam Y, Biasoli I, Lossos IS, Anderson MW, Morais JC, Spector N. Lack of association of tumor-associated macrophages with clinical outcome in patients with classical Hodgkin's lymphoma. Ann Oncol. 2012;23:736–42. doi:10.1093/annonc/mdr157. PMID:21602260.
49. Cencini E, Fabbri A, Rigacci L, Lazzi S, Gini G, Cox MC, Mancuso S, Abruzzese E, Kovalchuk S, Goteri G, et al. Evaluation of the prognostic role of tumour-associated macrophages in newly diagnosed classical Hodgkin lymphoma and correlation with early FDG-PET assessment. Hematol Oncol. 2017;35:69–78. doi:10.1002/hon.2249. PMID:26251194.
50. Steidl C, Farinha P, Gascoyne RD. Macrophages predict treatment outcome in Hodgkin's lymphoma. Haematologica. 2011;96:186–9. doi:10.3324/haematol.2010.033316. PMID:21282720.
51. Ruella M, Klichinsky M, Kenderian SS, Shestova O, Ziober A, Kraft DO, Feldman M, Wasik MA, June CH, Gill S. Overcoming the Immunosuppressive Tumor Microenvironment of Hodgkin Lymphoma Using Chimeric Antigen Receptor T Cells. Cancer Discov. 2017;7:1154–67. doi:10.1158/2159-8290.CD-16-0850.
52. Herbst H, Foss HD, Samol J, Araujo I, Klotzbach H, Krause H, Agathanggelou A, Niedobitek G, Stein H. Frequent expression of

interleukin-10 by Epstein-Barr virus-harboring tumor cells of Hodgkin's disease. Blood. 1996;87:2918–29. PMID:8639912.
53. Morales O, Mrizak D, François V, Mustapha R, Miroux C, Depil S, Decouvelaere A-V, Lionne-Huyghe P, Auriault C, de Launoit Y, et al. Epstein-Barr virus infection induces an increase of T regulatory type 1 cells in Hodgkin lymphoma patients. Br J Haematol. 2014;166:875–90. doi:10.1111/bjh.12980. PMID:25041527.
54. Chapman AL, Rickinson AB, Thomas WA, Jarrett RF, Crocker J, Lee SP. Epstein-Barr virus-specific cytotoxic T lymphocyte responses in the blood and tumor site of Hodgkin's disease patients: implications for a T-cell-based therapy. Cancer Res. 2001;61:6219–26. PMID:11507075.
55. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW, editors. WHO classification of tumours of haematopoietic and lymphoid tissues. 4th ed. Lyon, France: IARC Press; 2008.
56. Barros MHM, Hassan R, Niedobitek G. Disease patterns in pediatric classical Hodgkin lymphoma: a report from a developing area in Brazil. Hematol Oncol. 2011;29:190–5. doi:10.1002/hon.984. PMID:21374695.
57. Vera-Lozada G, Scholl V, Barros MHM, Sisti D, Guescini M, Stocchi V, Stefanoff CG, Hassan R. Analysis of biological and technical variability in gene expression assays from formalin-fixed paraffin-embedded classical Hodgkin lymphomas. Exp Mol Pathol. 2014;97:433–9. doi:10.1016/j.yexmp.2014.09.014. PMID:25236575.
58. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3:1101–8. doi:10.1038/nprot.2008.73. PMID:18546601.
59. Barros MHM, Hauck F, Dreyer JH, Kempkes B, Niedobitek G. Macrophage polarisation: an immunohistochemical approach for identifying M1 and M2 macrophages. PloS One. 2013;8:e80908. doi:10.1371/journal.pone.0080908. PMID:24260507.
60. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med. 1989;170:2081–95. doi:10.1084/jem.170.6.2081. PMID:2531194.