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

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**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 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/mm², 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+MAC+ (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.

**Introduction**

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

Inter-individual differences in IL10 production have a hereditary component estimated in 75%, mainly due to the effect of promoter polymorphisms, 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. Furthermore, genetic modifiers upstream of the transcription initiation site have been described. 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. 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. 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. 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. Associations of IL10 promoter polymorphisms with clinical outcome have been described in some studies with adult cHL patients, while in others this association has not been found. Moreover, an analysis of the...
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 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. 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% 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 $\chi^2$ test). Genotypic frequencies are described in Table 1. In view of the complete linkage of −819C/T and −592C/A SNPs, 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 $2^{-\Delta C_q} \pm -2.515 \pm 1.531$ SD] were higher than the observed in reactive follicular hyperplasia (RFH) lymph nodes [mean $2^{-\Delta C_q} \pm -3.757 \pm 1.235$ SD] ($P = 0.001$; Student’s t test) (Fig. 1A).

In the cHL group, −1082GG genotype was associated with lower *IL10* mRNA expression [mean $2^{-\Delta C_q} \pm 2.009$ SD] than AG+AA genotypes ($2^{-\Delta C_q} \pm 3.246 \pm 1.392$) ($P = 0.014$; 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^−ΔCq > −2.243, median of the group) presented more frequently with B symptoms (64.3% vs. 35.7% in low expression patients; $P = 0.013; \chi^2$ 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.

| Variable (cHL cases) | Number of Events | HR (Exp(b)) univar. | CI (95%) of univar. | P-value | HR (Exp(b)) multivar. | CI (95%) of multivar. | P-value |
|----------------------|-----------------|---------------------|-------------------|---------|-----------------------|----------------------|---------|
| −1082IL10            |                 |                     |                   |         |                       |                      |         |
| GG                   | 0/16            | 0.495               | 0.239             | 0.939   | 0.005                 | 0.005                |         |
| AG                   | 10/39           | 10/39               |                      |         | 0.031                 | 0.032                | 0.005   |
| AA                   | 10/33           | 0/16                | 0.095             | 0.001   | 0.691                 | 0.013                | 0.005   |
| −592IL10             |                 |                     |                   |         |                       |                      |         |
| AA                   | 5/10            | 2.350               | 1.278             | 4.291   | 0.009                 | 0.007                | 0.019   |
| ACC                  | 8/31            | 6/46                |                   |         | 2.362                 | 1.157                | 4.860   |
| CC                   | 6/46            | 6/46                |                   |         | 0.032                 | 0.034                | 0.005   |
| −592IL10             |                 |                     |                   |         |                       |                      |         |
| ACC                  | 9/43            | 0.853               | 0.347             | 2.068   | 0.072                 | 0.072                | 0.051   |
| ATA                  | 10/43           | 10/43               |                   |         | 1.505                 | 0.521                | 4.700   |
| −592IL10             |                 |                     |                   |         | 0.035                 | 0.035                | 0.045   |
| ACC                  | 9/31            | 0.560               | 0.231             | 1.375   | 0.020                 | 0.020                | 0.005   |

1P-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.
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 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).](image1)

![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).](image2)
ciliated, 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’s 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, −1082 GG 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, including 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.

| Variable                        | HR (Exp(b)) | Lower  | Upper  | P-value |
|---------------------------------|-------------|--------|--------|---------|
| **(A)** Extranodal sites        |             |        |        |         |
| High number of Granzyme B cells | 6.641       | 1.820  | 21.684 | 0.006   |
| Leukopenia                      | 4.179       | 1.218  | 12.122 | 0.021   |
| Mixed cellularity               | 2.822       | 0.848  | 8.756  | 0.086   |
| −1082GG genotype               | 3.637       | 1.242  | 10.076 | 0.020   |
| −1082GG genotype               | 0.054       | 0.000  | 0.511  | 0.005   |

This multivariate analysis was performed with 74 patients.

| Variable                        | HR (Exp(b)) | Lower  | Upper  | P-value |
|---------------------------------|-------------|--------|--------|---------|
| **(B)** Extranodal sites        |             |        |        |         |
| High number of Granzyme B cells | 6.209       | 1.504  | 25.200 | 0.008   |
| Leukopenia                      | 3.313       | 0.823  | 13.535 | 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.

| Variable                        | HR (Exp(b)) | Lower  | Upper  | P-value |
|---------------------------------|-------------|--------|--------|---------|
| **(C)** Extranodal sites        |             |        |        |         |
| High number of Granzyme B cells | 6.853       | 1.622  | 26.888 | 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.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).

### 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/mm², and median: 49 vs. 108 cells/mm²; 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/mm², 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+/CD8+ over CD8+ and TET1+ cells; and MAF+/CD8+ and TET1+ 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+/TET1+ 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.
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 and the presence of the −592AA genotype.

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...
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 immunosuppressor 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 draining 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{37} The −1082A allele was also correlated with high IL10 expression in whole blood from rheumatoid arthritis patients stimulated in vitro with lipopolysaccharide,\textsuperscript{38} in peripheral blood mononuclear cells stimulated with ConA,\textsuperscript{39} as well as in plasma of healthy individuals.\textsuperscript{40}

Discrepancy with the studies that found −1082GG genotypes associated with high expression levels\textsuperscript{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)\textsuperscript{3} where epigenetic mechanisms might be modulated by the diverse microenvironment stimuli.

In agreement with this 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.\textsuperscript{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,\textsuperscript{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.\textsuperscript{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.\textsuperscript{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\textsuperscript{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.\textsuperscript{9} 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,\textsuperscript{45–47} while some others failed to disclose such association.\textsuperscript{48,49} This discrepancy may be due to differences in immunohistochemical biomarkers and scoring systems.\textsuperscript{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.\textsuperscript{51} 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.\textsuperscript{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\textsuperscript{52,53} and a role of IL10 expression by H-RS cells in the evasion of viral-directed cytotoxicity,\textsuperscript{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,\textsuperscript{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 and immunohistochemical (IHC) characterization. Latent EBV infection has been investigated previously in all cHL cases by EBER-ISH hybridization. All patients were treated according to adriamycin-based standard pediatric protocols, as described. 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 micromet- sized 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. 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 TechnologiesTM, 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.

Immunohistochemical characterization of tumor microenvironment

A tissue microarray (TMA) block was built as described previously. The immune cells from the TME were identified by single or double immunohistochemistry, as described previously, with primary antibodies described in Table S5. Briefly, immunodetection was performed using ZytoChem Plus HRP polymer kit (Zytomed Systems, catalog number: POLHRP-100), employing dianaminobenzidine (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. The computer assisted cell quantification was performed with the image analysis software HISTO (Biomas, Erlangen, Germany), as described previously.

The identification of all MAF+ cells were performed by single IHC, while the specific identification of MAF+ macrophages was performed by double IHC. MAF+ inflammatory cells and MAF+ macrophages were expressed as absolute number/mm². 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 –values. 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.

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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; M.H.M.B. reviewed cases, performed and supervised TME characterization and reviewed the manuscript; G.N. and the interleukin-10 receptor. Annu Rev Immunol. 2001;19:683–700. doi:10.1146/annurev.immunol.19.1.683. PMID:11244051.

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.

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