Effect of *Interleukin-10* Gene Polymorphisms on Clinical Outcome of Patients with Aggressive Non-Hodgkin's Lymphoma: An Exploratory Study

Dieter Kube,[1] Thanh-Duc Hua,[1] Frederike von Bonin,[1] Nils Schoof,[1] Samira Zeynalova,[3] Marita Klöss,[3] Daniela Gocht,[1] Bernd Potthoff,[1] Mladen Tzvetkov,[2] Jürgen Brockmöller,[2] Markus Löffler,[3] Michael Pfreundschuh,[4] and Lorenz Trümper[1]

Abstract

Purpose: Current chemotherapy can achieve high response rates in aggressive non-Hodgkin's lymphoma (NHL), but the factors that influence regression and survival remain unknown. The present exploratory study tested the hypothesis whether interleukin-10 (IL-10) polymorphisms predict clinical outcome, leukocytopenia, or infectivity during therapy. IL-10 was chosen because immune alterations are a major risk factor for NHL, and IL-10 is a cytokine involved in inflammatory processes associated with clinical outcome.

Experimental Design: Five hundred patients with aggressive NHL treated with CHOP/CHOEP were analyzed for *IL-10* gene polymorphisms, including distal loci -7400InDel, -6752AT (rs6676671), and -6208CG (rs10494879) in comparison with proximal loci -3538AT (rs1800890), -1087AG (rs1800896), and -597AC (rs1800872) according to the incidence and outcome of the lymphoma.

Results: No differences in allele frequencies or haplotypes were found comparing a cohort of patients with aggressive NHL/diffuse large B-cell lymphoma with a healthy control group. Patients with aggressive NHL characterized by IL-10$_{-7400DelDel}$ had shorter overall survival periods compared with the other genotypes (P = 0.004). The 3-year rate is 43.4% for IL-10$_{-7400DelDel}$ and 73.4% for IL-10$_{-7400InIn}$ and IL-10$_{-7400InDel}$ together. A significant increased risk for event-free survival is found for carriers of the genotype IL-10$_{-6752TT-6208CC-3538AA}$ (P = 0.047). Multivariate analysis of IL-10$_{-7400}$ gene variation in relation to overall survival adjusted to international prognostic index revealed a relative risk of 1.9 for carriers of IL-10$_{-7400Del-Del}$ (P = 0.037). No associations were found analyzing diffuse large B-cell lymphoma patients separately.

Conclusion: Our results indicate that *IL-10* gene variations could be associated to the clinical course of aggressive NHL, which points out the importance of host factors and respective genetic elements for treatment response.

Authors' Affiliations: Departments of [1]Hematology and Oncology and [2]Pharmacology and Toxicology, Medical School of the Georg-August-University, Göttingen, Germany; [3]University of Leipzig, Institute of Medical Informatics, Statistics and Epidemiology, Leipzig, Germany; [4]Department of Internal Medicine I, Saarland University, Homburg/Saar for the German High-Grade NHL Study Group, Homburg/Saar, Germany

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Current address for D. Gocht: Institute of Zoology, Georg-August-University Göttingen, Göttingen, Germany. Current address for B. Potthoff: Amgen Research GmbH, Regensburg, Germany.

Requests for reprints: Dieter Kube, Universitätsmedizin der Georg-August-Universität Göttingen, Zentrum für Innere Medizin, Abteilung Hämatologie und Onkologie, 37099 Göttingen, Germany. Phone: 49-551-395307; Fax: 49-551-398587; E-mail: dkube@gwdg.de.

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With conventional chemotherapy, long-term remission can be achieved in approximately 50% of patients with disseminated "aggressive" non-Hodgkin's lymphoma (NHL). The disease incidence is increasing, but etiologic factors contributing to this phenomenon remain still largely unknown. Although it is a curable disease, many patients do not achieve complete remission or they relapse after conventional chemotherapy. Tumor- and host-related variables, differences in the response to therapy, may be related to genetic factors of the host (1, 2).

Deregulated components of the immune system, for example, cytokines, may be linked to the incidence and clinical course of lymphomas by the development of acute or chronic inflammatory reactions at tumor sites. Deregulated expression of defined subsets of cytokines was found to be associated with the transformation of lymphatic cells either as autocrine growth factors for the transformed cells or as factors rebuilding the tumor microenvironment, likely affecting tumor progression and dissemination (3).

Some reports support the hypothesis that common genetic variants in immune and inflammatory response genes can

affect the risk and clinical outcome of NHL as well as side effects of therapy like infections or hematotoxicity (for review, see refs. 4–6).

The magnitude and profile of immune responses are regulated to a large extent by cytokines. The extent to which cytokines secretion varies between individuals with consequent variations in the intensity of a given immune response could be defined in part by regulatory gene variations. Several regulatory genetic elements associated with differences in cytokine secretion have been identified in genes coding for cytokines, in part also associated with disease outcome (for review, see refs. 7, 8). These regulatory polymorphisms are therefore thought to be partially responsible for interindividual differences to cope with a given challenge to the immune system. Specific cytokine genotypes may be beneficial by creating a "proinflammatory" phenotype that may predispose to chronic inflammatory diseases or to a more severe form of inflammatory disease with a worse clinical outcome. However, the mechanisms underlying differences in immune response between individuals are complex but include inherited genetic variation.

Interleukin-10 (IL-10) is an important immunoregulatory cytokine in man. IL-10 is part of a balanced network of cytokines and can be cancer promoting (immunosuppressive; stimulation of cell proliferation) or cancer inhibiting (antiangiogenic; refs. 9–11). IL-10 is produced by several cells including normal and neoplastic B cells, stimulated monocytes/macrophages, and subsets of T cells. IL-10 has been implicated in certain infectious diseases, autoimmunity, transplantation tolerance, and tumorigenesis (for review, see also refs. 4, 9, 12, 13).

Polymorphisms in the *IL-10* 5′-flanking region genetically affect interindividual differences in IL-10 production (14–23). Variable associations between IL-10 production capacity and either the *IL-10* microsatellite alleles, single nucleotide polymorphisms (SNP), or SNP haplotypes in the 7-kb *IL-10* 5′-flanking region have been reported (14, 16–18, 23).

In most studies, the major proximal haplotypes GCC, ACC, or ATA formed by SNPs IL-10$_{-1087AG}$, IL-10$_{-824CT}$, and IL-10$_{-597AC}$ were found to be related to the *in vitro* IL-10 production capacity. The ATA haplotype was described as IL-10 low producer. (14, 16–18, 23) Several studies have reported that these proximal *IL-10* promoter polymorphisms may be related with increased risk of a diverse range of diseases (reviewed in ref. 4). This indicates that genetic variations within the *IL-10* gene locus are relevant *in vivo*.

Recent reports provided evidence that a risk to develop NHL or the clinical outcome of patients suffering from diffuse large B-cell lymphoma (DLBCL) might be related to certain *IL-10* promoter gene variations. In one study, it was reported that proximal genotypes or haplotypes with low IL-10 expression are a risk factor for aggressive lymphoma, whereas a second study suggest that genotypes of high expression potential are a risk factor for developing lymphoma in patients with AIDS (24, 25). An InterLymph epidemiologic multicenter study described the IL-10$_{-3538A}$ regulatory SNP to be associated with for increased risk to develop NHL (26). This, however, was not verified in a subgroup from Germany/Heidelberg (27). In our study, allele frequencies of lymphoma patients are comparable with those of unmatched healthy controls (28). A French study (GELA) showed that in DLBCL patients the IL-10$_{-1087G}$ allele may be a risk factor for disease susceptibility, but this could not be verified in a cohort from Scandinavia (29, 30). In addition, no correlation to any clinical variables was found as described in the GELA study (29, 30). Analyzing the *IL-10* gene loci at -3538 (A/T), -1354 (A/G), -824 (C/T), and -597 (A/C), we did not find any difference in overall survival (OS) or event-free survival (EFS) for NHL patients (28). However, these studies differed to some extent in terms of age range, lymphoma subtype, modest study size, and partially insufficient power.

The aim of this study was to analyze distal gene variations within the 5′-flanking region of the *IL-10* gene in a large, representative, equally treated cohort of patients suffering

Table 1. Clinical characteristics and diagnosis of patients with aggressive NHL

Patient characteristics	All patients in NHL-B1/B2 trials (*N* = 1,399)	All patients analyzed for IL-10 gene variations (*n* = 500)	DLBCL patients analyzed for IL10 gene variations (*n* = 319)
Sex			
Male	789 (56)	280 (56)	180 (56)
Female	610 (44)	220 (44)	139 (44)
Age, median (min; max)	60 (18; 75)	62 (23; 75)	62 (23; 75)
Serum LDH >N	316 (23)	123 (25)	85 (27)
Age >60 y	689 (49)	273 (55)	178 (56)
Performance status ECOG >1	163 (12)	65 (13)	46 (14)
Ann Arbor stage III/IV	567 (41)	202 (40)	122 (38)
No. extranodal sites ≥2	276 (20)	104 (21)	61 (19)
IPI*			
Low (IPI = 0, 1)	840 (60)	280 (56)	173 (54)
Low intermediate (IPI = 2)	250 (18)	99 (20)	66 (21)
High intermediate (IPI = 3)	170 (12)	67 (13)	46 (14)
High (IPI = 4, 5)	139 (10)	54 (11)	34 (11)
Bulky tumor (≥7.5 cm) present	467 (33)	164 (33)	109 (34)
B symptoms	402 (29)	142 (28)	86 (27)
Extranodal involvement	698 (50)	252 (50)	153 (48)

NOTE: Values in table expressed as total number of patients (%), unless otherwise indicated. For additional information about the histology of patients with aggressive NHL, refer to Supplementary File 1.
*LDH >N, age >60 years, ECOG >1, stage III/IV, and number of extranodal sites ≥2.

Table 2. Genotype frequency and genotype distribution of the *IL-10* polymorphisms in patients with aggressive NHL (n = 500) and healthy control subjects (n = 236)

Genotypes	Control, *n* (%)	NHL patients, *n* (%)	*P*
-7400InDel			
InIn	150 (64)	305 (61)	0.995*
InDel	72 (31)	178 (36)	0.110 †
DelDel ‡	14 (6)	17 (3)	0.505§
-6752AT			
AA	91 (39)	176 (35)	0.399*
AT	113 (48)	250 (50)	0.655 †
TT ‡	32 (14)	74 (15)	0.376§
-6208CG			
CC ‡	44 (19)	80 (16)	0.928*
CG	110 (47)	262 (52)	0.371 †
GG	82 (35)	158 (32)	0.395§
-3538AT			
AA ‡	33 (14)	74 (15)	0.962*
AT	122 (52)	249 (50)	0.769 †
TT	81 (34)	177 (35)	0.775§
-1087AG			
AA	67 (28)	134 (27)	0.766*
AG	108 (46)	253 (51)	0.333 †
GG ‡	61 (26)	113 (23)	0.651§
-597AC			
AA ‡	14 (6)	26 (5)	0.436*
AC	98 (42)	196 (39)	0.683 †
CC	124 (53)	278 (56)	0.437§

*Genic comparison (allele).
† Minor homozygotes vs. heterozygotes + major homozygotes.
‡ Minor genotype.
§ Major homozygotes vs. heterozygote + minor homozygotes.

from aggressive NHL and their role in predisposing an individual to lower remission rates, OS, or shorter periods of EFS and whether these associations are distinctive for DLBCL subtypes. The comparison of these gene variations with clinical variables such as EFS and OS revealed that distal regulatory gene variations of the *IL-10* gene are related to some extent to poor prognosis of patients with aggressive NHL.

Materials and Methods

Patients and treatment. Lymphoma patients included into this study were from the NHL-B1/B2 study from the German NHL Study Group as described recently (Supplementary File 1; refs. 31, 32). The study was conducted in accordance with the Declaration of Helsinki. The protocol was approved by the ethics review committee of each participating center. All patients gave written informed consent. Patients were eligible if they had previously untreated, biopsy-confirmed aggressive NHL according to the Revised European-American Lymphoma Classification (translated into the WHO classification).

In this analysis, we included 500 patients from NHL-B1 and NHL-B2 studies. Three hundred and ninety-seven patients within this cohort were already genotyped for proximal SNPs at -1087A/G (rs1800896), -597A/C (rs1800872), and -3538A/T (rs1800890) as published recently (28). Clinical characteristics of the 500 patients eligible for this study are shown in Table 1 and are representative for all 1,399 NHL-B1 and NHL-B2 patients. The respective histology presented in Supplementary File 1 is based on a blinded central pathology review by a panel of expert hematopathologists.

The control group included 236 healthy blood donors. All samples were taken with no regard to sex or age, and donors were free of any chronic diseases.

Genotyping analyses. Blood samples and DNA isolation, multiplex PCR, and Taqman real-time PCR were done.

DNA from 236 unrelated healthy blood donors was included into this analysis as described previously (23, 33). DNA samples from 500 lymphoma patients were isolated by the same procedure and were described previously (23, 28, 34).

For the analysis of the genetic polymorphisms of the *IL-10* 5′-flanking region, a multiplex assay was used as described recently (23, 28, 33). Within the multiplex assay, the -7400InDel gene variation was analyzed as well as SNPs at -6752A/T (rs6676671), -6208C/G (rs10494879), -597A/C (rs1800872), and -3538A/T (rs1800890). In addition, the SNPs at -1087A/G (rs1800896), -6752A/T (rs6676671), -6208C/G (rs10494879), -597A/C (rs1800872), and -3538A/T (rs1800890) were analyzed by Taqman SNP genotyping assays (for details, see Supplementary File 2).

Statistical analysis. For the analysis of the *IL-10* polymorphisms, 500 patients were selected from the NHL-B1/B2 study population, considering the factors of the international prognostic index [IPI; age >60 years, lactate dehydrogenase (LDH) >N, Eastern Cooperative Oncology Group (ECOG) >1, stage III/IV, >1 extranodal involvement], bulky disease, and B symptoms to be representative for the NHL-B1/B2 trial population.

Genetic data were analyzed using GENEPOP software. Analysis included tests for Hardy-Weinberg equilibrium and genotypic and allelic differentiation between healthy controls and lymphoma patients. Haplotype analysis was done using Arlequin software and http://www.bioinf.mdc-berlin.de/projects/hap/. WHO grades for leukocytopenia and infection, genic, genotypic, and allelic differentiation between groups were analyzed using the χ^2 test and, if required, Fisher's exact test.

EFS was defined as time from first day of therapy to progressive disease under therapy or failure to achieve complete remission or CR unconfirmed (that is, no change or partial remission associated with additional therapy), additional therapy in excess of that prescribed in the protocol, relapse or death from any cause, whichever came first. OS was defined as time from first day of therapy to death from any cause.

Patients without an event in EFS or OS were censored at the last day with valid information for the respective endpoint. EFS and OS were estimated according to Kaplan-Meier and compared by log-rank test.

Multivariate analyses were done with the use of Cox proportional hazards models to estimate hazard ratios for evolving an event. Nominal significance level was at 0.05 (two-sided). We are aware of the problem of multiple comparisons and therefore have chosen to extract the most prominent aspect. Statistical analyses were done with SPSS (version 11.5) software.

Results

IL-10 gene polymorphisms in patients with aggressive NHL and in healthy control subjects. The *IL-10* 5′-flanking gene variations at IL-10$_{-7400InDel}$, IL-10$_{-6752AT}$, IL-10$_{-6208CG}$, IL-10$_{-3538AT}$, IL-10$_{-1087AG}$, and IL-10$_{-597AC}$ were analyzed in 500 NHL patients. Allele frequencies, genotypes, and haplotypes were defined and compared with corresponding healthy controls. In Table 2, genic and genotypic data are summarized for the *IL-10* gene variations. For the IL-10$_{-7400InDel}$ gene variation only, the genotype IL-10$_{-7400DelDel}$ is less frequently present in the group of patients with NHL. However, this difference is not significant (P = 0.110). The testing showed that there are no significant differences between healthy controls and NHL patients in our study as well as for the other analyzed gene loci.

Our control group of 236 healthy blood donors was taken with no regard to sex or age, and donors were free of any chronic diseases. Therefore, this is not a classic case-control

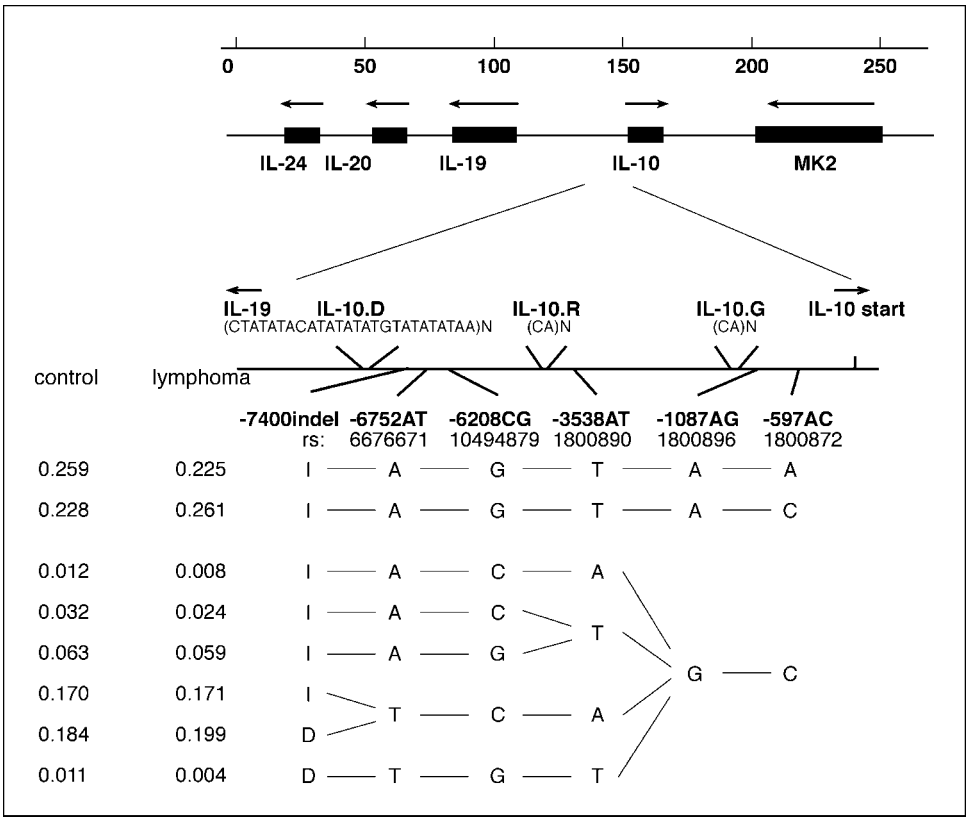

Fig. 1. Graphical view of the extended haplotypes of the 5′-flanking region of the *IL-10* gene. *IL-10* gene variations -7400, -6752, -6208, -3538, -1087, and -597 in relation to the IL-10 promoter microsatellites IL-10.R, IL-10.G, and IL-10.D and the ATG (start) of the *IL-10* gene as described in Kube et al. (22) and Mormann et al. (23). *Top,* schematic view of the *IL-10* family gene cluster on chromosome 1q31/32. The extended haplotype frequencies are shown for the healthy controls (control, $n = 236$) and the NHL patients (lymphoma, $n = 500$). No significant differences in haplotypes are found. Gene variations of -3538, -1087, and -597 have been genotyped in a previous study in 379 lymphoma patients.

study. However, a comparison of genotype distribution for IL-10$_{-3538AT}$ and IL-10$_{-1087AG}$ of our control group with published data from EPILymph Germany performing a classic case-control study revealed no relevant differences (Supplementary Table S1; refs. 26, 27). The Hardy-Weinberg test showed no significant differences between observed and suspected numbers of homozygotes or heterozygotes for the analyzed gene variations of the *IL-10* gene in both groups (Supplementary Table S1). The same is found for the subgroup of 319 DLBCL patients within our study (Supplementary Table S2).

Based on the presented genotyping data, respective haplotypes of the 5′-flanking region of the *IL-10* gene were estimated. All haplotypes with a frequency higher than 3% are shown in Fig. 1. Haplotype estimation was done for both healthy controls and NHL patients. Four major haplotypes are present: IAGTAA, IAGTAC, ITCAGC, and DTCAGC (IL-10 gene variations -7400, -6752, -6208, -3538, -1087, and -597). The IL-10$_{-7400Del}$ locus seems to be nearly exclusively linked with IL-10$_{-1087G}$. The further analysis of haplotypes reveals no significant differences between healthy controls and lymphoma patients.

IL-10 polymorphisms and aggressive NHL outcome. The clinical prognostic features incorporated in the IPI, including age, LDH level, performance status, clinical stage and number of extranodal sites, mostly reflect the disease extension and the patient's characteristics. Therefore, we compared gene variations of the 5′-flanking region of the *IL-10* gene with these clinical variables, including hematotoxicity or infections according to WHO grades. Among 500 NHL patients, no associations were found between *IL-10* gene variations and five single IPI prognostic factors (LDH >N, ECOG >1, extranodal involvement >1, stage >II, and age >60 years).

Infection WHO grade 3 or 4 during the first cycle of chemotherapy ranged between 0% for patients characterized by IL-10$_{-7400DelDel}$ and 5.6% carrying IL-10$_{-3538AA}$. However, these differences were not significant between analyzed genotypes (data not shown). Similar results were obtained when taking into account all cycles of chemotherapeutic treatment. The rate of cycles with infection grade 3 or 4 was also not significant.

Leukocytopenia as a variable of hematotoxicity of chemotherapy was analyzed in relation to *IL-10* gene variations. For the gene variation IL-10$_{-597AC}$, we observed a risk to develop leukocytopenia with WHO grade 3 or 4 for heterozygous patients. Leukocytopenia of WHO grades 3 and 4 was observed in 37% and 39% cycles for homozygous AA and CC patients, respectively, and in 45% cycles for heterozygous AC patients ($P = 0.002$). The rates of patients with leukocytopenia grades 3 and 4 were 38%, 31%, and 47% for patients with AA, CC, and AC, respectively ($P = 0.001$).

Univariate analysis of OS and EFS of 500 NHL patients in comparison with *IL-10* gene variations showed a significantly shorter OS for the *IL-10* genotype IL-10$_{-7400DelDel}$ (Table 3; Fig. 2A). The respective 3-year survival rates were significantly reduced. Within this period, only 43.4% of patients carrying IL-10$_{-7400DelDel}$ survived [95% confidence interval (95% CI), 19%, 68%], whereas 72.3% (95% CI, 67%, 77%) or 75.3% (95% CI, 69%, 82%) of patients carrying IL-10$_{-7400InIn}$ and IL-10$_{-7400InDel}$, respectively, had no event of death ($P = 0.009$). The OS rate for the IL-10$_{-7400DelDel}$ genotype is shown in more detail as a Kaplan-Meier plot in Fig. 2A. The OS difference between patients carrying IL-10$_{-7400DelDel}$ and patients with the other genotypes together was also significant ($P = 0.004$;

Table 3). The IL-10$_{-7400DelDel}$ genotype has been described recently as "IL-10 high producer" (35). The 3-year survival rates for patients with IL-10$_{-6752TT}$ was 64.6% (95% CI, 53%, 76%) and 73.7% (95% CI, 69%, 78%) for patients with IL-10$_{-6752AT}$ and IL-10$_{-6752AA}$ together with a borderline significance (P = 0.051; Table 3). The OS rate for the IL-10$_{-6752TT}$ genotype is also shown in more detail as a Kaplan-Meier plot in Fig. 2B. For IL-10$_{-6208CG}$, IL-10$_{-3538}$, IL-10$_{-1087}$, and IL-10$_{-597AC}$ gene variations, no significant shorter cumulative OS periods were observed (Table 3).

The analysis of EFS showed no significant differences when analyzing single loci. However, the *IL-10* genotypes IL-10$_{-7400DelDel}$ and IL-10$_{-6752TT}$ were characterized by a trend toward shorter EFS periods (P = 0.091 and 0.063, respectively; Fig. 2C and D).

A haplotypic analysis showed that almost all patients (16 of 17) characterized by a homozygous genotype IL-10$_{-7400Del}$ were also homozygous for IL-10$_{-6752T}$ as part of the haplotype DelTCAGC (IL-10 gene variations -7400, -6752, -6208, -3538, -1087, and -597) and have an unfavorable disease outcome.

When comparing OS or EFS of homozygous carriers for the TCA haplotype (IL-10 gene variations -6752, -6208, and -3538) with all other genotypes, a significant association with EFS (P = 0.047) and a borderline significance for OS (P = 0.064) were observed (Fig. 2E and F).

In a multivariate analysis, we investigated the effect of the genotype variations after adjusting for the five IPI risk factors (age >60 years, elevated pretreatment LDH, ECOG performance state >1, advanced stage III/IV, and >1 extranodal involvement). Only genotype IL-10$_{-7400DelDel}$ compared with IL-10$_{-7400InsIns}$ and IL-10$_{-740InslDel}$ had a clear trend of increasing risk (relative risk, 1.9) and was significant in OS (P = 0.037; Table 4). The estimated relative risk of 1.9 is comparable with that of the variables used in clinical practice.

Analysis of the subgroup of 319 DLBCL patients showed no significant differences between healthy controls and NHL patients (Supplementary Table S2). For the OS, the 3-year rate was 51% for patients carrying IL-10$_{-7400DelDel}$ versus 72% for patients carrying IL-10$_{-7400InIn}$ and IL-10$_{-7400InDel}$ (P = 0.096; small number of patients with IL-10$_{-7400DelDel}$; n = 11). For all other analyzed gene loci, no significant OS and EFS differences were observed.

Discussion

The role of inherited factors in the extent of IL-10 deregulation in malignant disorders is still controversial. The preliminary data obtained thus far indicate that additional larger studies of patients are required to confirm initial results in the understanding of the role of IL-10 in lymphoma development. We report one of the first analyses of the association between far distal gene variations of the *IL-10* 5′-flanking region and aggressive NHL within the thus far largest homogenously treated aggressive NHL patients group with 500 individuals representing the NHL-B1/B2 study from the DSHNHL study group. The results of the present exploratory study strongly support the hypothesis that genetic polymorphisms within the chromosomal locus 1q31/32 of the 5′-flanking region of the *IL-10* gene are associated with adverse prognostic factors and predict poor outcome of aggressive NHL. In this study, we show that patients suffering from aggressive NHL carrying the *IL-10* genotype

Table 3. OS and EFS of patients suffering from aggressive NHL in relation to gene variations of the *IL-10* gene 5′-flanking region

Genotype	3-y rate OS	*P*	3-y rate EFS	*P*	Genotype	3-y rate OS	*P*	3-y rate EFS	*P*
-7400									
InIn (n = 305)	72.3	*0.009*	59.1	0.190	InIn; InDel	73.4	*0.004*	60.4	0.091
InDel (n = 178)	75.3		62.5		DelDel	43.4		39.2	
DelDel (n = 17)	43.4		39.2						
-6752									
AA (n = 176)	72.8	0.143	60.3	0.166	AA; AT	73.7	0.051	60.8	0.064
AT (n = 250)	74.4		61.2		TT	64.6		53.2	
TT (n = 74)	64.6		53.2						
-6208									
CC (n = 80)	68.8	0.448	59.4	0.768	GG; CG	73.1	0.365	59.7	0.499
CG (n = 262)	74.4		59.9		CC	68.8		59.4	
GG (n = 158)	70.9		59.6						
-3538									
AA (n = 74)	66.3	0.172	56.2	0.217	AT; TT	73.5	0.116	60.3	0.137
AT (n = 249)	75.2		61.4		AA	66.3		56.2	
TT (n = 177)	71.0		58.7						
-1087									
AA (n = 134)	71.7	0.788	60.6	0.936	AA; AG	73.4	0.553	60.1	0.733
AG (n = 253)	74.2		59.8		GG	69.1		58.5	
GG (n = 113)	69.1		58.5						
-597									
AA (n = 26)	71.6	0.666	52.4	0.778	CC; AC	72.4	0.416	60.1	0.480
AC (n = 196)	72.7		59.5		AA	71.6		52.4	
CC (n = 278)	72.2		60.5						

NOTE: Patients characterized by the genotypes IL-10$_{-7400DelDel}$ or IL-10$_{-6752TT}$ had a poorer prognosis compared with the other genotypes respectively. Italic P values are significant. For continuative description of significant results, see also Fig. 2.

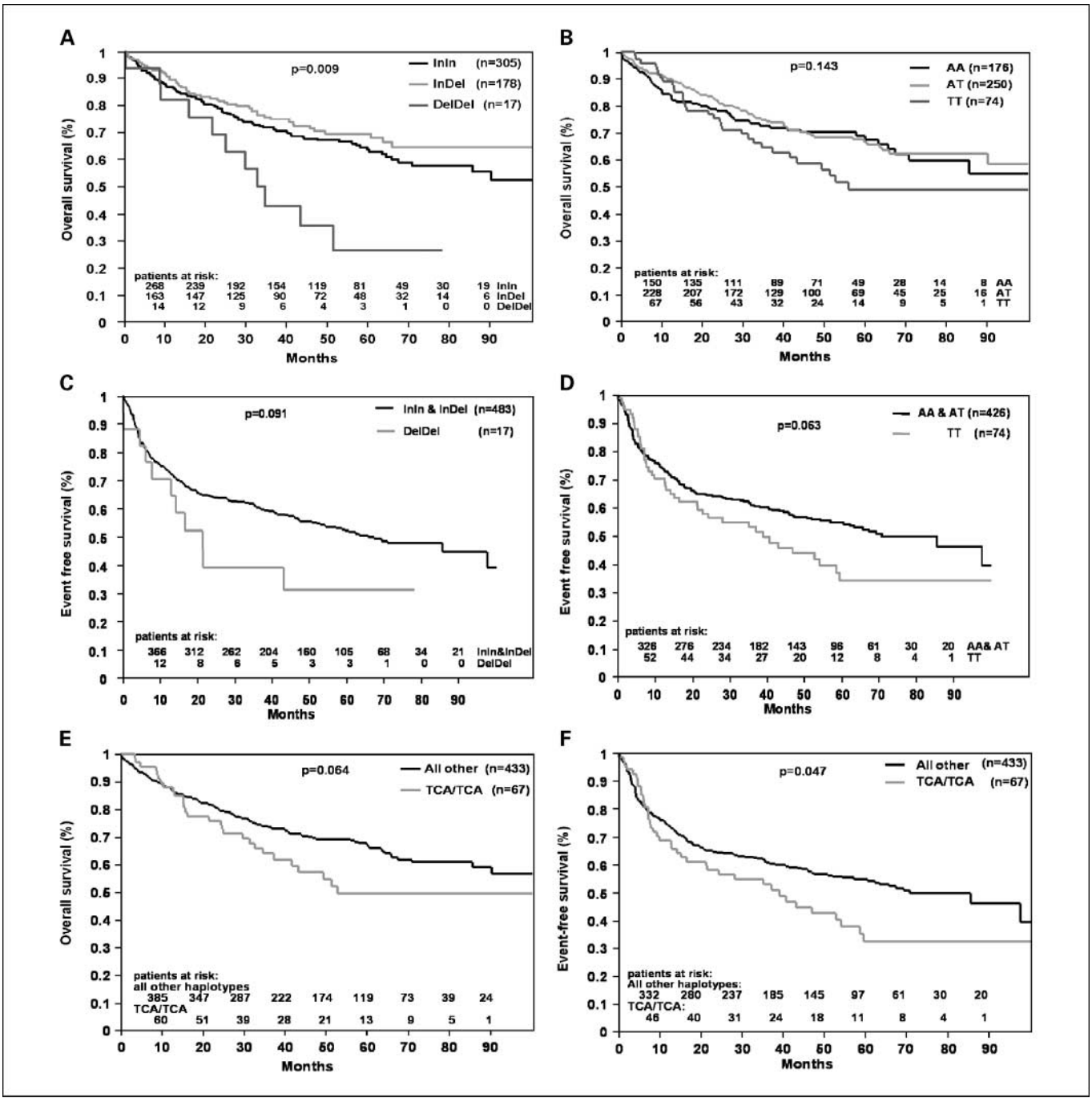

Fig. 2. OS and EFS of patients suffering from aggressive NHL in relation to gene variations of the *IL-10* gene 5′-flanking region. Comparison of genotypes for OS in (*A*) IL-10$_{-7400}$ ($P = 0.009$), (*B*) IL-10$_{-6752}$ ($P = 0.143$), and (*E*) IL-10$_{-6752,-6208,-3538}$ ($P = 0.064$) and EFS for (*C*) IL-10$_{-7400}$ ($P = 0.091$), (*D*) IL-10$_{-6752}$ ($P = 0.063$), and (*F*) IL-10$_{-6752,-6208,-3538}$ ($P = 0.047$). When comparing IL-10$_{-7400DelDel}$ with all other genotypes, the effect for OS is stronger ($P = 0.004$). For IL-10$_{-6752TT}$ compared with all other genotypes, the difference in OS is borderline significant ($P = 0.051$; for additional details, see also Table 3). *E* and *F,* OS and EFS for patients suffering from aggressive NHL compared with the homozygous genotype IL-10$_{-6752TT-6208CC-3538AA}$ (IL-10$_{TCA}$). *P* refers to the log-rank test. Patients at risk represent the number of patients who still can develop an event (OS or EFS) at defined time points (here 10-month interval monitored).

IL-10$_{-7400DelDel}$ have a shorter cumulative OS. In addition, homozygous carriers for TCA haplotypes (*IL-10* gene variations -6752, -6208, and -3538) have poor EFS.

The multivariate analysis of the most distal gene variation an insertion/deletion mutation at -7400 revealed a 1.9-fold increased risk for carriers of the deletion on both alleles to have a poor prognosis (OS; Table 4). This observed relative risk is comparable with those estimated by analyzing clinical variables (age >60 years and ECOG >1), which have a relative risk of 2.0 or 2.1, respectively.

The increased relative risk of carriers of -7400DelDel has to be discussed also in the context of the IL-10 production capacity of respective healthy carriers. In a recent work, we showed that this genotype is characterized by an extremely high IL-10 expression capacity after *in vitro* stimulation with lipopolysaccharide (35). The IL-10$_{-7400DelDel}$ genotype is rare

Table 4. Multivariate analysis of far distal IL-10$_{-7400}$ gene variations in relation to OS adjusted to IPI (Cox model)

Factor	Relative risk (95% CI)	*P*
LDH >N	1.7 (1.2-2.5)	0.005
Age > 60 y	2.0 (1.3-3.0)	0.001
ECOG > 1	2.1 (1.4-3.0)	<0.001
Stage III/IV	1.5 (1.0-2.1)	0.029
Extranodal involvement >1	1.0 (0.7-1.4)	0.888
DelDel vs InsIns, InsDel	1.9 (1.0-3.6)	0.037

in Caucasians as well as in other thus far analyzed geographic regions from central Africa or Vietnam (23).[5] This could suggest that additional gene variations probably in linkage disequilibrium to the described haplotype are important but not yet identified. Follow-up analysis will be needed to investigate additional gene variations across the chromosomal region 1q31/32 as suggested recently by Lan et al. (36). Several DNase I–hypersensitive sites on a conserved 40-kb region between *IL-19* and *IL-10* genes revealed three functional enhancer elements, which reflect changes of the chromatin structure associated with *IL-10* gene expression. Two of these enhancer elements with influence on IL-10 gene expression are located in comparable distances to the transcriptional start site of *IL-10* as the analyzed far distal gene variations (37–43). Whether these chromatin changes are also affected by far distal IL-10 gene variations and influence therefore the differential IL-10 expression level remains to be analyzed in the future.

Promoter polymorphisms, including so-called regulatory SNPs, have been subject to the most scrutiny, particularly with regard to possible influence on regulation of gene transcription. The mechanism behind this for the *IL-10* gene is still unknown probably because of the close proximity of -1087AG to the IL-10.G microsatellite; the IL-10.R microsatellite may affect IL-10 expression levels (17, 19). At this stage, the precise role of *IL-10* promoter gene variations, individually or as part of defined proximal or distal haplotypes, in determining IL-10 expression is still a subject awaiting answers, including the question of allele-specific regulation (44). For example, the haplotype TCA (IL-10$_{-6752T\text{-}6208C\text{-}3538A}$) associated with poor EFS of NHL patients showed in *in vitro* experiments an intermediate response to lipopolysaccharide (23, 35). In addition, patients carrying IL-10$_{-3538AA}$ are characterized by an increased risk to display an IPI >2 (P = 0.025 and 0.033, respectively) in both aggressive NHL and respective DLBCL patients. Patients with IPI >2 are about twice more often (>20%) homozygous for IL-10$_{-3538A}$ (for additional details, see Supplementary Table S3). However, after Bonferroni correction for multiple testing, these differences would be not longer significant. This observation that patients homozygous for IL-10$_{-3538AA}$ may have a higher risk for IPI >2 and that carriers of IL-10$_{-7400DelDel}$ and IL-10$_{6752TT}$ are characterized by shorter cumulative OS may support the hypothesis that specific cytokine gene variations or polymorphisms in regulatory regions of cytokine genes are markers of tumor progression. Our finding of an increased risk to develop leukocytopenia WHO grades 3 and 4 when carrying both IL-10$_{-597A}$ and IL-10$_{-597C}$ alleles adds some new aspect but does not fit to the current understanding of IL-10 production capacity and therefore remains to be elucidated by additional studies and *in vitro* experiments.

The clinical outcome of patients suffering from DLBCL was not related to proximal *IL-10* promoter gene variations as described by other investigations. There was no significant difference in OS and EFS in the subgroup of DLBCL patients, but remarkably the genotype IL-10$_{-7400DelDel}$ had a clear trend in OS. Apparently these conflicting data may in part reflect the pleiotropic functions of IL-10 as cancer promoter or inhibitor in relation to the biological growth patterns of the lymphoma subgroups (11). Furthermore, differences in cancer karyotype complexity are also defining the disease state. In addition, antigenic and nonantigenic stimuli may be affecting the IL-10 expression by the lymphoma cells or their microenvironment (45).

Gene expression data have started to delineate the known molecular heterogeneity of aggressive NHL into distinct molecular entities but underlined the role of host response factors in some subtypes of these lymphomas (46–48). Bringing together molecular classifications, epidemiologic findings with well-designed clinical trials and respective *in vitro* analysis will help us understand the regulatory role of inherited IL-10 expression in the pathogenesis of lymphoma (49). Our major finding about an association between the *IL-10* genotype IL-10$_{-7400DelDel}$ and the significant shorter cumulative OS or that of the haplotype TCA (IL-10$_{-6752T\text{-}6208C\text{-}3538A}$) with poor EFS of NHL patients is, however, not yet suitable as a prognostic factor in routine use.

In this work, we have chosen to extract the most prominent clinical aspect and defined a new focused hypothesis for further validation study: IL-10$_{-7400DelDel}$ or the haplotype TCA (IL-10$_{-6752T\text{-}6208C\text{-}3538A}$) could be a risk factor for poor clinical outcome for patients with aggressive NHL. This hypothesis can now be verified in independent patient cohort, for example, within the DSHNHL RICOVER study, or even in cohorts from other study groups with comparable expert hematopathologist review and treatment regimen.

[5] Kube et al., unpublished observations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Kaiser U, Uebelacker I, Abel U, et al. Randomized study to evaluate the use of high-dose therapy as part of primary treatment for "aggressive" lymphoma. J Clin Oncol 2002;20:4413–9.
2. Wunderlich A, Kloess M, Reiser M, et al. Practicability and acute haematological toxicity of 2- and 3-weekly CHOP and CHOEP chemotherapy for aggressive non-Hodgkin's lymphoma: results from the NHL-B trial of the German High-Grade Non-Hodgkin's Lymphoma Study Group (DSHNHL). Ann Oncol 2003;14:881–93.
3. Lin WW, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest 2007;117:1175–83.
4. Hollegaard MV, Bidwell JL. Cytokine gene polymorphism in human disease: on-line databases, supplement 3. Genes Immun 2006;7:269–76.
5. Howell WM, Rose-Zerilli MJ. Interleukin-10 polymorphisms, cancer susceptibility and prognosis. Fam Cancer 2006;5:143–9.
6. Lossos IS, Morgensztern D. Prognostic biomarkers

in diffuse large B-cell lymphoma. J Clin Oncol 2006;24:995–1007.
7. Bidwell J, Keen L, Gallagher G, et al. Cytokine gene polymorphism in human disease: on-line databases, supplement 1. Genes Immun 2001;2:61–70.
8. Haukim N, Bidwell JL, Smith AJP, et al. Cytokine gene polymorphism in human disease: on-line databases, supplement 2. Genes Immun 2002;3:313–30.
9. 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.
10. Cervenak L, Morbidelli L, Donati D, et al. Abolished angiogenicity and tumorigenicity of Burkitt lymphoma by interleukin-10. Blood 2000;96:2568–73.
11. Mocellin S, Marincola FM, Young HA. Interleukin-10 and the immune response against cancer: a counterpoint. J Leukoc Biol 2005;78:1043–51.
12. Blay JY, Burdin N, Rousset F, et al. Serum interleukin-10 in non-Hodgkin's lymphoma: a prognostic factor. Blood 1993;82:2169–74.
13. Benjamin D, Park CD, Sharma V. Human B cell interleukin 10. Leuk Lymphoma 1994;12:205–10.
14. Turner D, Williams D, Sankaran D, Lazarus M, Sinnott P, Hutchinson I. An investigation of polymorphism in the interleukin-10 gene promoter. Eur J Immunogenet 1997;24:1–8.
15. Westendorp R, Langermans J, Huizinga T, et al. Genetic influence on cytokine production and fatal meningococcal disease. Lancet 1997;349:170–3.
16. Gibson AW, Edberg JC, Wu J, Westendorp RG, Huizinga TW, Kimberly RP. Novel single nucleotide polymorphisms in the distal IL-10 promoter affect IL-10 production and enhance the risk of systemic lupus erythematosus. J Immunol 2001;166:3915–22.
17. Eskdale J, Gallagher G, Verweij C, Keijsers V, Westendorp R, Huizinga T. Interleukin 10 secretion in relation to human IL-10 locus haplotypes. Proc Natl Acad Sci U S A 1998;95:9465–70.
18. Crawley E, Kay R, Sillibourne J, Hutchinson I, Woo P. Polymorphic haplotypes of the IL-10 5′ flanking region determine variable IL-10 transcription and are associated with particular phenotypes of juvenile rheumatoid arthritis. Arthritis Rheum 1999;42:1101–8.
19. Eskdale J, Kube D, Tesch H, Gallagher G. Mapping of the human IL10 gene and further characterization of the 5′ flanking sequence. Immunogenetics 1997;46:120–8.
20. Gallagher G, Dickensheets H, Eskdale J, et al. Cloning, expression and initial characterisation of interleukin-19 (IL-19), a novel homologue of human interleukin-10 (IL-10). Genes Immun 2000;1:442–50.
21. D'Alfonso S, Rampi M, Rolando V, Giordano M, Momigliano Richiardi P. New polymorphisms in the IL-10 promoter region. Genes Immun 2000;1:231–3.
22. Kube D, Rieth H, Eskdale J, Kremsner P, Gallagher G. Structural characterisation of the distal 5′ flanking region of the human interleukin-10 gene. Genes Immun 2001;2:181–90.
23. Mormann M, Rieth H, Hua TD, et al. 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.
24. Cunningham L, Chapman C, Dunstan R, Bell M, Joske D. Polymorphisms in the interleukin 10 gene promoter are associated with susceptibility to aggressive non-Hodgkin's lymphoma. Leuk Lymphoma 2003;44:251–5.
25. Breen EC, Boscardin WJ, Detels R, et al. Non-Hodgkin's B cell lymphoma in persons with acquired immunodeficiency syndrome is associated with increased serum levels of IL10, or the IL10 promoter -592 C/C genotype. Clin Immunol 2003;109:119–29.
26. Rothman N, Skibola CF, Wang SS, et al. Genetic variation in TNF and IL10 and risk of non-Hodgkin lymphoma: a report from the InterLymph Consortium. Lancet Oncol 2006;7:27–38.
27. Nieters A, Beckmann L, Deeg E, Becker N. Gene polymorphisms in Toll-like receptors, interleukin-10, and interleukin-10 receptor α and lymphoma risk. Genes Immun 2006;7:615–24.
28. Kube D, Hua TD, Kloss M, et al. The interleukin-10 gene promoter polymorphism -1087AG does not correlate with clinical outcome in non-Hodgkin's lymphoma. Genes Immun 2007;8:164–7.
29. Lech-Maranda E, Baseggio L, Bienvenu J, et al. Interleukin-10 gene promoter polymorphisms influence the clinical outcome of diffuse large B-cell lymphoma. Blood 2004;103:3529–34.
30. Berglund M, Thunberg U, Roos G, Rosenquist R, Enblad G. The interleukin-10 gene promoter polymorphism (-1082) does not correlate with clinical outcome in diffuse large B-cell lymphoma. Blood 2005; 105:4894–5; author reply 5.
31. Pfreundschuh M, Trumper L, Kloess M, et al. Two-weekly or 3-weekly CHOP chemotherapy with or without etoposide for the treatment of elderly patients with aggressive lymphomas: results of the NHL-B2 trial of the DSHNHL. Blood 2004;104:634–41.
32. Pfreundschuh M, Trumper L, Kloess M, et al. Two-weekly or 3-weekly CHOP chemotherapy with or without etoposide for the treatment of young patients with good-prognosis (normal LDH) aggressive lymphomas: results of the NHL-B1 trial of the DSHNHL. Blood 2004;104:626–33.
33. Kube D, Mörmann M, Tomiuk J, Hua T, Kremsner P, Vockerodt M. Simultaneous analysis of interleukin-10 gene microsatellites and single-nucleotide polymorphisms in parallel with tumour necrosis factor and interferon-γ short tandem repeats by fluorescence-based polymerase chain reaction. Genes Immun 2003;4:459–68.
34. Wojnowski L, Kulle B, Schirmer M, et al. NAD(P)H oxidase and multidrug resistance protein genetic polymorphisms are associated with doxorubicin-induced cardiotoxicity. Circulation 2005;112:3754–62.
35. Rieth H, Mormann M, Luty AJ, et al. 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.
36. Lan Q, Zheng T, Rothman N, et al. Cytokine polymorphisms in the Th1/Th2 pathway and susceptibility to non-Hodgkin lymphoma. Blood 2006;107:4101–8.
37. Jones EA, Flavell RA. Distal enhancer elements transcribe intergenic RNA in the IL-10 family gene cluster. J Immunol 2005;175:7437–46.
38. Im SH, Hueber A, Monticelli S, Kang KH, Rao A. Chromatin-level regulation of the IL10 gene in T cells. J Biol Chem 2004;279:46818–25.
39. Zhang X, Edwards JP, Mosser DM. Dynamic and transient remodeling of the macrophage IL-10 promoter during transcription. J Immunol 2006;177: 1282–8.
40. Wang ZY, Sato H, Kusam S, Sehra S, Toney LM, Dent AL. Regulation of IL-10 gene expression in Th2 cells by Jun proteins. J Immunol 2005;174:2098–105.
41. Shoemaker J, Saraiva M, O'Garra A. GATA-3 directly remodels the IL-10 locus independently of IL-4 in $CD4^+$ T cells. J Immunol 2006;176:3470–9.
42. Saraiva M, Christensen JR, Tsytsykova AV, et al. Identification of a macrophage-specific chromatin signature in the IL-10 locus. J Immunol 2005;175: 1041–6.
43. Lucas M, Zhang X, Prasanna V, Mosser DM. ERK activation following macrophage FcγR ligation leads to chromatin modifications at the IL-10 locus. J Immunol 2005;175:469–77.
44. Kurreeman FA, Schonkeren JJ, Heijmans BT, Toes RE, Huizinga TW. Transcription of the IL10 gene reveals allele-specific regulation at the mRNA level. Hum Mol Genet 2004;13:1755–62.
45. Farinha P, Masoudi H, Skinnider BF, et al. Analysis of multiple biomarkers shows that lymphoma-associated macrophage (LAM) content is an independent predictor of survival in follicular lymphoma (FL). Blood 2005;106:2169–74.
46. Hummel M, Bentink S, Berger H, et al. A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. N Engl J Med 2006;354: 2419–30.
47. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 2000;403:503–11.
48. Rosenwald A, Wright G, Leroy K, et al. Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favorable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma. J Exp Med 2003;198:851–62.
49. Kwiatkowski D. Genetic dissection of the molecular pathogenesis of severe infection. Intensive Care Med 2000;26:S89–97.