Mechanisms of aberrant GATA-3 expression in classical Hodgkin lymphoma and its consequences for the cytokine profile of Hodgkin and Reed/Sternberg cells

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Abstract

The transcription factor network in Hodgkin lymphoma (HL) represents a unique composition of proteins found in no other hematopoietic cell. Among these factors, an aberrant expression of the T cell transcription factor GATA-3 is observed in the B cell-derived Hodgkin and Reed/Sternberg (HRS) tumor cells. Herein, we elucidated the regulation and function of this factor in HL. We demonstrate binding of NFκB and Notch-1, two factors with deregulated activity in HL, to GATA-3 promoter elements. Interference with NFκB and Notch-1 activity led to decreased GATA-3 expression, indicating a dependency of deregulated GATA-3 expression on these transcription factors. Downregulation of GATA-3 in HL cell lines demonstrated its role in the regulation of IL-5, IL-13, STAT-4 and further genes. A correlation between GATA-3 and IL-13 expression was confirmed for HRS cells in HL tissues. Thus, GATA-3 shapes the cytokine expression and signalling that is typical for HL. Conclusively, aberrant GATA-3 expression in HRS cells is stimulated by the deregulated constitutive activity of NFκB and Notch-1, indicating a complex network of deregulated transcription factors in these cells. GATA-3 activity significantly contributes to the typical cytokine secretion of and signalling in HRS cells, which presumably plays an essential role in HL pathogenesis.
Introduction

Hodgkin lymphoma (HL) is one of the most frequent malignant lymphomas in the Western world. Characteristic of classical HL (cHL) is the occurrence of a small number of the typical Hodgkin and Reed/Sternberg (HRS) tumor cells among a mixed cellular infiltrate that is essential for their survival. In this tumor microenvironment CD4^+ helper and regulatory T cells make up the largest populations of infiltrating hematopoietic cells. Even though HRS cells have largely lost their B cell phenotype, they derive in nearly all cases from germinal center B cells. HRS cells express a variety of markers that are not associated with B cells (e.g. granzyme B, CCL17, STAT-5). A previous gene expression study of cHL cell lines performed by us revealed that HRS cells also aberrantly express the T-cell transcription factor GATA-3, which was not expressed by normal mature B cells or any of several other types of B cell lymphomas included in that analysis.

GATA-3 is an essential transcription factor for early T cell development. During T_H cell development GATA-3 primes cells for the T_H2 phenotype by shutting down production of factors promoting T_H1 development (e.g. IFNγ), and by opening T_H2 target loci by direct promoter activation (e.g. IL-5, IL-13). Lately, GATA-3 overexpression has been associated with the outgrowth of pancreatic cancer cells, whereas its expression in breast cancer seems to be a favourable prognostic factor.

Similarly to GATA-3, Notch-1 is an inducer of T_H2 development, yet, relying on the presence of GATA-3 for this function. Moreover, direct promoter transactivation of GATA-3 by Notch-1 has been demonstrated in murine CD4^+ T cells. Notch-1 is also expressed in HRS cells, and Notch-1 activity in cHL leads to accelerated growth and reduced apoptosis, and contributes to the suppression of B cell gene expression in HRS cells. Activation of Notch-1 in HRS cells appears to be mediated through its ligand Jagged-1, which is expressed on bystander cells in the tumor micromilieu of cHL.
Constitutive NFκB activity is another hallmark of HRS cells and plays a central role for the pathogenesis of HL.\textsuperscript{19,20} Multiple factors, including genetic lesions in genes encoding NFκB inhibitory factors, such as NFKBIA and TNFAIP3, contribute to the strong NFκB activity.\textsuperscript{1,21,22} NFκB activity was first linked to GATA-3 expression in airway epithelial cells when p50 knockout mice were unable to express GATA-3.\textsuperscript{23} Furthermore, these mice were unable to generate T_{H}2 cells.

Here, we wanted to study the mechanisms for the aberrant GATA-3 expression in HRS cells and its consequences for gene expression and in particular for the cytokine signalling network in cHL. We applied functional assays to elucidate whether GATA-3 is part of a transcription factor network driven by NFκB and Notch-1 activity in cHL.

\textbf{Materials and Methods}

\textbf{Cell lines and primary tissue}

Cell lines were purchased from DSMZ (Braunschweig, Germany) and cultured in RPMI 1640 supplemented with 10\% fetal calf serum (Biochrom; Berlin, Germany), 2 mM glutamine (Invitrogen; Groningen, The Netherlands) and 1\% penicillin G/streptomycin sulfate (Invitrogen). U-HO1 cells were cultured as above but in IMDM. Fresh frozen lymph nodes from patients diagnosed with cHL were acquired from the collection at the Senckenberg Institute of Pathology, University of Frankfurt/Main, Germany. The Internal Review Board of the University of Frankfurt approved the usage of the biopsy material.

\textbf{IL-13 suppression assays}

For details, see Suppl. Methods.

\textbf{Subcellular fractionation}

For details, see Suppl. Methods.
Gene expression arrays and statistical analysis

The details are given in Suppl. Methods.

Immunoblots

Immunoblots were performed by standard methods (see Suppl. Methods).

Lentivirus vector production

Lentiviral particles overexpressing IκBα superrepressor were generated as previously described. For further details and shRNA sequences, see Suppl. Methods.

Semi-quantitative RT-PCR

RT-PCR was performed on total RNA prepared with RNeasy Micro Kit (Qiagen). Following DNase I treatment, equal amounts of RNA were reverse transcribed using Sensiscript RT (Qiagen) and random hexamers. PCR amplification was performed as previously described. A minimum number of cycles was performed to obtain a clear signal upon agarose gel electrophoresis within the linear amplification phase. The following specific primer pairs were used: GATA-3: 5′-ggcctcagcactctcat-3′ & 5′-cactctttctgctg-3′; GAPDH: 5′-cacagtccatgccatcac-3′ & 5′-caccaccctgttgctg-3′; STAT-4: 5′-atgtctcagtgaatcaagt-3′ & 5′-ctgtctttctgaactgaag-3′; IL-13: 5′-caccgagaaccagaagctc-3′ & 5′-ggcagaatccgctcag-3′; IL-5: 5′-atgaggtgtctgtcatttt-3′ & 5′-ggcctgcaatgatatc-3′.

Immunohistochemistry

We performed immunohistochemical stainings for GATA-3 and IL-13 for 35 cases of cHL. The stainings for GATA-3 failed for two cases. Among the evaluable 33 cases 26 (79%) were scored as positive for GATA-3 expression in HRS cells. The IL-13 staining was more difficult
to apply and evaluate. One case had to be excluded because of inconsistent results of the IL-13 staining. Finally, we ended up with a collection of 16 cases with evaluable stainings for GATA-3 and IL-13. For IL-13 evaluation, only a broad estimation of positive HRS cells was possible, because of many IL-13 positive non-HRS cells and a considerable background staining of this secreted molecule. For GATA-3 evaluation, in a first collection of 10 cases (including 4 consistently negative) also a broad estimation of positivity was performed. When staining six additional cases, the frequency of GATA-3 positivity was determined more precisely by counting positive and negative HRS cells (8-42 per case). Importantly, the immunostainings for GATA-3 and IL-13 were evaluated in a blinded fashion. The following antibodies were used for immunohistochemical stainings on frozen tissue sections: anti-GATA-3 (Santa Cruz Biotechnology; #sc-268) 1:50 dilution, anti-IL-13 (Abcam; Ab16219, Cambridge, UK) 1:100 dilution. For antigen retrieval sections were fixed with acetone for 10 minutes. Detection of antibody binding, was performed with the REAL-AP system (Dako REAL™ Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse K5005; Hamburg, Germany) and the bridge goat-immunglobulin biotin (Rabbit, Dako E0466) were used. Incubation with the primary antibodies was 30 minutes at room temperature for both antibodies.

**Enzyme linked immunosorbent assay**

A human IL-13 ELISA Kit was used according to the manufacturer's protocol (Perbio, Rockford, USA). In brief, cell culture supernatants of cells grown for three, five, or seven days were separated from the cells by centrifugation for 5 minutes at 300 x g and 4°C. Triplicate measurements of each supernatant were performed.
MTT assay
Following lentiviral transduction or treatment with IL-13 binding protein cells were seeded at a density of $10^4$ cells per well of a 96-well plate and grown for the indicated time points. Cell viability was assayed at indicated times in triplicate wells using the CellTiter96 AQueous One Solution Cell Proliferation Assay (Promega; Mannheim, Germany).

Chromatin immunoprecipitation (ChIP)
ChIP-IT express kit (Active Motif, Rixensart, Belgium) was performed on HL cell lines according to the manufacturer's protocol. PCR conditions were performed as described for semi-quantitative RT-PCR. The following primer sequences were used to detect Notch-1 and NFκB binding sites in the GATA-3 promoter according to Genbank entry NC_000010: -3762: 5'-cgctcctttcccttcccccttt-3'; -3382: 5'-gggatggagaagggcaacc-3'; -561: 5'-tgctgagccagcccggct-3'; -386: 5'-gggagacctggatgagcccc-3'; -306: 5'-gcagaattgcagagtcgtcg-3'; +158: 5'-gggtgtgcccgttgacgacg-3'.

Cell sorting
Two to ten days post transduction virally infected cells were sorted, using a DIVA cell sorter (Becton Dickinson). Besides the expression of shRNAs, the lentiviral particles used herein also express the green fluorescent protein (GFP) in a second expression cassette under the spleen focus-forming virus (SFFV) promoter. Therefore, equal numbers of living, i.e. propidium iodide negative, cells were sorted according to their GFP expression for further analysis.

Cell cycle distribution flow cytometry
Cells were harvested at indicated time points, fixed in 70% ethanol, washed, stained with propidium iodide and analyzed for their cell cycle profile.
Quantitative real-time PCR

Quantitative Taqman PCR was performed on cDNA reverse transcribed from RNA of same numbers of GFP-positive sorted cells. Real-time PCR was performed on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) using Taqman Gene Expression assays Hs00231122_m1 for GATA-3, Hs01062014_m1 for Notch-1, Hs99999903_m1 for actin, Hs00154109_m1 for Birc3, Hs00164932_m1 for ICAM-1, Hs00236874_m1 for Lta (Applied Biosystems) and Taqman Universal PCR master mix NoAmp Erase UNG (Applied Biosystems) according to manufacturer’s protocols. For quantitative PCR of ChIP samples conditions as before were used with four custom designed assays generated by Applied Biosystems: Assay -523NFκB (assay ID AIQJARO), assay -88NFκB (assay ID AIVI3F0), assay upstream Notch-1 (assay ID AI89I9B), assay downstream Notch-1 (assay ID AIAAYE4).

Mutational analysis of Notch-1

The details of the mutational analysis of Notch-1 are given in Suppl. Methods.

Results

GATA-3 is expressed in cHL cell lines and effectively downregulated by lentiviral-mediated shRNA expression

Based on differential gene expression studies of cHL cell lines and various other B cell lymphomas and normal B cells, we previously detected consistent and specific expression of multiple non-B cell specific genes in the HL cell lines.⁸ Among these upregulated mRNAs the T cell-specific transcription factor GATA-3 was found, and its expression was validated on protein level.⁸ Atayar and colleagues confirmed this finding in primary HL tissue
demonstrating that in two-third of HL cases analysed by them, 25 to >75% of HRS cells were positive for GATA-3.25

To obtain a more comprehensive picture of GATA-3 expression, seven HL cell lines was analyzed for protein abundance (Fig. 1A). GATA-3 expression was strongest in the cHL cell lines U-HO1 and L-428, whereas a moderate expression was seen in KM-H2 and L-1236. No expression was detected in the EBV-positive L-591, the T cell-derived HDLM-2, and the lymphocyte-predominant HL cell line DEV. The surprising lack of GATA-3 in the T cell derived HDLM-2 was previously described.8

To embark on the function of GATA-3 we used shRNA to knockdown its expression in HL cell lines. Two different shRNAs directed against the GATA-3 sequence were cloned into lentiviral vectors, which also encode GFP. Transduction of cHL cell lines expressing GATA-3, namely U-HO1, L-428, KM-H2, and L-1236, with shRNA expressing viral particles resulted in transduction efficiencies of 30% in KM-H2, 50% in U-HO1 and L-428 and up to 80% in L-1236 three days post infection (as measured by the fraction of GFP-positive cells). The four transduced lines responded to both shRNAs directed against GATA-3 with reduced GATA-3 protein levels as early as three days (L-1236), four days (U-HO1), or five days post infection (L-428, KM-H2) compared to a non-functional scrambled shRNA (SCR) (Fig. 1B). The amount of reduced protein levels was heterogenous. Both shRNAs induced a strong downregulation of GATA-3 protein to undetectable levels in KM-H2 and L-1236, whereas the siGATA-3 #1 was more potent than siGATA-3 #2 in L-428 and U-HO1 cells. The inhibition of GATA-3 by shRNA knockdown was also observed on mRNA levels in the four transduced cell lines, indicating that the knockdown is not limited to interference on translational but also extends to the transcriptional level (Fig. 1C).

GATA-3 inhibition leads to downregulation of IL-5, IL-13, and STAT-4
To test the consequences of GATA-3 downregulation on typical GATA-3 target genes we extended the semi-quantitative RT-PCR analysis to three known GATA-3 targets. The direct transactivation of the IL-5 and IL-13 genes by GATA-3 was first suggested in 1995, whereas a suppressive effect of GATA-3 expression on STAT-4 has been described in developing Th1 and Th17 cells.

In KM-H2 cells, loss of GATA-3 concomitantly led to significantly diminished amounts of STAT-4, as opposed to the inverse correlation described for T cells (Fig. 2A). IL-5 could not be amplified from KM-H2 cells, as expected from our gene expression profiles that showed a lack of IL-5 expression in this cell line. The expression of IL-13 in KM-H2 cells has been controversially discussed in the literature. We could not detect any expression of IL-13 in KM-H2 cells, in line with the data from Atayar and colleagues.

The L-428 cell line expresses all three downstream target genes. While IL-5 and STAT-4 expression was diminished to undetectable levels upon downregulation of GATA-3 (Fig. 2B), IL-13 expression remained unaltered.

In U-HO1 cells IL-13 was strongly downregulated following reduction of GATA-3 with one of the two GATA-3-specific shRNAs (Fig. 2C). Additionally, STAT-4 expression was no longer detectable in these cells even when GATA-3 was only incompletely knocked down (lane 4, Fig. 2C). IL-5 could not be detected in U-HO1 cells by RT-PCR.

The L-1236 cell line lacks IL-5 expression. IL-13 expression in these cells is robustly decreased when GATA-3 expression is depleted. Although STAT-4 was expected to be expressed in L-1236 cells we were unable to amplify its mRNA even when extended PCR conditions were applied. Taken together, GATA-3 activity directly influences various cytokines and activators in a number of different cHL cell lines.

To obtain a more comprehensive overview of GATA-3 regulated genes in cHL cell lines, we performed a genechip analysis comparing gene expression in GATA-3-specific shRNA-transduced L-1236 and U-HO1 cells compared to samples transduced with vectors
encoding scrambled shRNAs. The downregulation of GATA-3 in this experiment was relatively moderate, but statistically significant, with a downregulation by a factor of 3.5- to 7.2-fold in L-1236 cells and between 5.5- to 7.5-fold in U-HO1 cells according to a quantitative Taqman pre-test assay (not shown). According to the genechip analysis GATA-3 transcript levels were downregulated to 55% for UHO-1 cells and 69% for L-1236. Regarding the three GATA-3 targets (IL-5, IL-13 and STAT-4) analysed in detail by specific quantitative RT-PCR (see above), these three genes were not detected above background levels in L-1236 cells, so that the genechips were not informative for their regulation. Signals for IL-13 and STAT-4 were detected at low level in UHO-1 cells, but no significant regulation was observed, although this was consistently seen in multiple earlier specific RT-PCR experiments. This may be explainable by the only moderate downregulation of GATA-3 in this experiment. Importantly, however, in U-HO1 cells IL-5 was the most downregulated gene (2.05-fold). Thus, we can add IL-5 to the genes that are regulated by GATA-3 in UHO-1 cells.

The gene expression profiles of all siGATA-3 and siSCR treated samples for U-HO1 and L-1236 cells were analyzed for genes with up- or downregulated expression levels of more than 1.5-fold and false discovery rates (FDR) of less than 0.25 (Table S1). In this analysis the siGATA-3 treated samples of U-HO1 and L-1236 were together compared to the control samples, to focus on genes consistently regulated by GATA-3 (directly or indirectly) in both lines. Ten genes were found to be downregulated and 18 genes were upregulated. Notably, the known functions of the 28 consistent GATA-3 targets include apoptosis regulation, regulation of transcription, cell cycle control and others, indicating that GATA-3 not only regulates components of cytokine signalling but is involved in the regulation of multiple cellular processes in HRS cells.
GATA-3 abundance correlates with IL-13 expression in HL

Because the connection between IL-13 and GATA-3 was partly inconsistent in the cHL cell lines (observed in two of the four cell lines) we wanted to clarify whether a correlation between these two factors exists in HL, especially since a pro-survival activity has been attributed to IL-13 expression in HL. Therefore, we performed immunohistochemical stainings for both proteins for 35 cases representing the histological subtypes nodular sclerosis (NS) or mixed cellularity (MC) of cHL, of which 16 cases with evaluable stainings for both GATA-3 and IL-13 were included in the final collection (see Methods; Table S2). All cases were at least 60% positive in HRS cells for IL-13. Generally, the cases with enhanced positivity of 80-90% also showed elevated levels of GATA-3 positivity of at least 70%. We applied the Pearson’s correlation test to determine whether there is a statistically significant correlation between nuclear GATA-3 positivity and IL-13 expression in our collection (Fig. 3). Under the hypothesis of a T distribution, the correlation is significant with a p-value of 0.0025. The correlation of 0.619 indicates that there is a linear correlation between GATA-3 and IL-13 expression.

As three of the cases – for unknown reasons – showed only cytoplasmatic localization of GATA-3 and three further cases cytoplasmatic as well as nuclear positivity (Table S2), we analyzed the GATA-3 expressing cell lines for subcellular localization of this factor (Fig. S1A). GATA-3 was exclusively found in the nucleus in all four HL cell lines and not seen in the cytoplasm, which is also true when GATA-3 was knocked down by shRNAs in L-1236 cells (Fig. S1B).

To further evaluate the association between GATA-3 and IL-13 under cell culture conditions we transduced HL cell lines with shRNAs for GATA-3 and monitored IL-13 levels in the cell culture medium by ELISA (Fig. 4A). Protein lysates from the cells corresponding to the cell culture medium samples used for ELISA were assayed for GATA-3 protein expression in immunoblots (Fig. 4B). GATA-3 proteins significantly decreased as early as
three days post infection whereas secreted IL-13 amounts started to notably diminish at around five days post infection in L-1236 cells. At seven days post infection IL-13 levels were reduced from 325 pg/ml to approximately 200 pg/ml for both shRNA constructs and hence to about 60% of the cytokine level in the cell culture medium of scrambled shRNA transduced cells. Expectedly, L-428 did not show reduced levels of secreted IL-13 as a consequence of GATA-3 depletion (data not shown) in accordance with the results of the semi-quantitative RT-PCR (Fig. 2B). Moreover, the amount of IL-13 in KM-H2 culture medium was below detection level (7 pg/ml) in the ELISA (data not shown) and consequently verifies the results of our semi-quantitative RT-PCR (Fig. 2A) where no IL-13 could be amplified due to lack of IL-13 mRNA. In conclusion, IL-13 and GATA-3 correlate in vitro and in vivo in HL indicating that GATA-3 contributes to IL-13 expression in HRS cells, although HL cell lines show a heterogenous picture.

**GATA-3 expression has no pro-survival effect in HL**

Since we had established that IL-13 expression is dependent on GATA-3 activity in HL and IL-13 cytokine expression is often described as a pro-survival factor for HRS cells,30,32,33 we embarked on the question whether GATA-3 likewise has a pro-survival and hence anti-apoptotic function in HRS cells. Upon transduction of L1236 cells with shRNAs targeting GATA-3 the amount of metabolically active and thus healthy, dividing cells was measured in an MTT assay (Fig. 5A). Even though a decrease of viable cells following GATA-3 knockdown was observed 3, 5, and 7 days post infection - when compared to mock infected cells set as 100% viable cells - the effect was not significantly different from cells treated with non-functional scrambled control shRNA. The effects in MTT assays measured for L-428 and KM-H2 cells were similar to the results in L-1236 cells (data not shown). To further validate these findings, we performed immunoblots on transduced cells and analyzed the cells for cleaved caspase 3 as a marker for apoptotic cells. L-1236 cells did not show any signs of
apoptosis up to one week post lentiviral transduction (Fig. 5B) even though GATA-3 was fully depleted. The results for L-428 and KM-H2 were in accordance with the findings for L-1236 (data not shown). Therefore, GATA-3 does not influence the survival of HL cells in our cell culture models. However, depletion of IL-13 activity as previously described\(^3\) reduced the viability of L-1236 cells (Fig. S2A) whereas none of the other three cell lines reacted on the IL-13 binding protein. The reduced amount of metabolically active cells is caused by apoptosis due to an increased number of cells with sub-G1 content (Fig. S2B). Therefore, GATA-3 may not be the only factor involved in IL-13 regulation in HL and additional interference with other unknown upstream IL-13 regulators would be needed to produce similar effects as seen with IL-13 binding protein.

**GATA-3 regulatory elements are occupied by Notch-1 and NFκB proteins**

Even though we could assign a functional role for GATA-3 in cHL, the mechanisms by which its expression is regulated was hitherto enigmatic. Therefore, we analyzed putative regulatory elements surrounding the GATA-3 promoter. Because of the reported linkage of GATA-3 expression with NFκB activity in murine airway epithelial cells and direct GATA-3 promoter activation by Notch-1 in murine T cells,\(^{16,23}\) we focussed on potential binding sites for these two factors in the human GATA-3 gene. We used the known binding sequences GGGRNNYYCC for NFκB\(^{35}\) and TGGGAA for Notch-1/CSL\(^{36}\) and identified several putative binding sites within or near the GATA-3 promoter according to the Genbank entry NC_000010. Three Notch-1 binding sites at -3523, -153, and -144 relative to the translational start site set as +1 were identified. Two NFκB sites were identified, one at -88 and one within intron 1 at position -523 (Fig. 6A). We performed ChIP assays to clarify whether any of these sites is occupied by Notch-1 and/or NFκB. After antibody treatment and consecutive PCRs we observed that Notch-1 robustly occupied the more 5’ binding site termed 'upstream Notch-1' as well as the more 3’ located site 'downstream Notch-1' in L-428 and U-HO1 cells (Fig.
6B). However, the PCR band intensity generated after precipitation with Notch-1 antibody was weak for the downstream binding site in L-428 cells.

The ChIP for NFκB binding showed that the –523 site within intron 1 was occupied by the p65 activator subunit of NFκB in all three cell lines (Fig. 6C). In L-428 and L-1236 the binding was less pronounced than in KM-H2 cells. Additionally, precipitation with the IgG isotype negative control antibody resulted in faint bands in these two lines indicating some background signal. The -88 NFκB binding site was only weakly occupied compared to negative control samples by p65 in L-1236 cells; the other two cell lines lacked p65 binding. To validate these findings quantitatively, we performed additional ChIP experiments with L-428 and U-HO1 cells and evaluated the transcription factor binding by Taqman PCR. These studies nicely validated for both cell lines the binding of Notch-1 and NFκB to their respective binding sites (Fig. S3). Therefore, we demonstrated binding of both transcription factors at various promoter and/or regulatory elements in the GATA-3 gene in HL cell lines, indicating that GATA-3 expression might be regulated by these transcription factors.

**Notch-1 and NFκB activity induce GATA-3 expression in HL**

To elucidate the effect of Notch-1 and NFκB binding for GATA-3 expression we interfered with the activity of Notch-1 by shRNA knockdown and diminished NFκB activity by expression of an IκBα superrepressor. Successful reduction of NFκB activity was validated by strong downregulation (at least four-fold) of the three typical NFκB target genes ICAM-1, Birc3, and Lta in three of the four transduced cell lines, and at least a moderate downregulation (1.5- to 4-fold) in KM-H2 cells (Fig 7A). As a consequence of this decreased NFκB activity also GATA-3 expression was consistently reduced in all four cell lines. In L-428 and L-1236 cells GATA-3 mRNA was downregulated by a factor of about 1.5-fold
whereas in KM-H2 and U-HO1 cell lines GATA-3 expression was reduced by 2.5- to three-fold. Therefore, NFκB activity regulates GATA-3 expression in cHL cell lines.

To modulate Notch-1 activity two different shRNA sequences were delivered via lentiviral transduction into cHL cells expressing Notch-1. To verify whether our cHL cell lines do express sufficient levels of Notch-1 as previously published37 we performed immunoblots on Jurkat T cells and on various cHL cell lines (Fig. 7B). HDLM-2, KM-H2, L-428, and U-HO1 cells expressed Notch-1 protein even stronger than the Jurkat leukemia cells. L-1236 cells had a weak expression similar to that of Jurkat cells. Hence, three cell lines, namely KM-H2, L-428, and U-HO1 expressed Notch-1 and GATA-3 at sufficient levels and could thus be used for shRNA assays. Notch-1 expression could be efficiently downregulated at least four-fold in all three cell lines with siN-1 #1 shRNA as determined by quantitative Taqman mRNA target gene measurement (Fig. 7C). siN-1 #2 shRNA led to 2.5- to three-fold downregulation of Notch-1 in U-HO1 cells. In all three cell lines we observed that as a consequence of altered Notch-1 expression also GATA-3 decreased to 2.5- to 3-fold when cells were transduced with siN-1 #1 shRNA against Notch-1. ShRNA siN-1 #2 had similar effects on GATA-3 expression when used in U-HO1 cells (Fig. 7C). Moreover, in immunoblots we detected that following Notch-1 protein level reduction also GATA-3 protein expression was drastically diminished in the cell lines L-428 and U-HO1 when siNotch-1 #1 or siNotch-1 #2 were used (Fig. 7D). Conclusively, besides the influence of NFκB activity on GATA-3 expression we could demonstrate that the T cell transcription factor Notch-1 also modulates the expression of GATA-3 in HL derived cell lines.

Discussion

HRS cells are B cell-derived in nearly all cases, but these cells have largely lost their B-lineage specific gene expression program and regularly express a variety of non-B cell markers,2 including GATA-3,8,25 which is in the hematopoietic system specifically expressed
in T cells, and among mature B cell lymphomas only found in HRS cells of cHL.\textsuperscript{8} Herein, we studied consequences of the aberrant GATA-3 expression in HRS cells and mechanisms for its deregulated expression. Three known GATA-3 target genes (IL-5, IL-13 and STAT-4) were analyzed specifically for altered expression following GATA-3 depletion. We could not analyze each of the three genes in each of the three cell lines, because the HL lines showed a surprisingly heterogenous expression pattern of IL-5, IL-13 and STAT-4. Importantly, however, with one exception (IL-13 in L-428), downregulation of GATA-3 led a consistent downregulation of the three genes in all instances where they were initially expressed. The finding that STAT-4 is positively regulated by GATA-3 expression in HL was unexpected and in contrast to previous findings in T\textsubscript{H1} and T\textsubscript{H17} cells.\textsuperscript{28,29} However, we consistently observed a positive regulation of STAT-4 by GATA-3 in three cell lines which indicates that its regulation in HRS cells seems to be inverse to the situation in normal T cells. The lack of responsiveness of L-428 regarding IL-13 transcript levels upon GATA-3 downregulation indicates that in this cell line IL-13 expression is regulated by a set of transcription factors that overcome the need for GATA-3. The consequences of GATA-3 expression for IL-13 expression was further analyzed at the protein level, showing that GATA-3 downregulation in L-1236 cells caused a reduced secretion of IL-13 into the culture medium.

The connection between IL-13 and GATA-3 was further studied on the protein level in vivo. Although most of the cases expressed both GATA-3 and IL-13, we could nevertheless identify a statistically significant correlation between the fraction of GATA-3-positive and IL-13-positive HRS cells in the HL cases. This indicated that also in vivo, GATA-3 is involved in the regulation of IL-13 expression and is an important, albeit not the only, positive modulator of its expression level.

Previously, a pro-survival effect was appointed to IL-13 in HRS cells,\textsuperscript{30,32,33} which we could confirm in L-1236 cells (Fig. S2). Therefore, we tested the effect of GATA-3 knockdown on survival and apoptosis induction in HL cell lines. However, neither by
measuring cell viability in an MTT assay, nor by immunoblot analysis for cleaved caspase-3, we could detect clear signs of apoptosis in any of the three HL lines analyzed. Thus, the partial downregulation of IL-13 by downregulation of GATA-3 is not strong enough to translate into a direct apoptotic effect for the HL cell line cells. Main functions of GATA-3 in HRS cells may relate to the modulation of the microenvironment by influencing cytokine signalling pathways, which can not be tested in HL cell lines growing in vitro in the absence of the typical HL micromilieu.

A genechip analysis revealed that GATA-3 regulates multiple further genes in HRS cells. Therefore, GATA-3 effects in HRS cells are not only limited to cytokine expression and signalling but extends to numerous other cellular functions.

Experiments performed to reveal the mechanisms for the aberrant expression of GATA-3 in HRS cells showed that the NFκB and Notch-1 transcription factors reside at binding sites in the vicinity of the transcriptional start site of GATA-3. The NFκB site within intron 1 is robustly bound by p65 activator subunits of NFκB in four cell lines, and the –88NFκB site in exon 2 was bound by NFκB in two of the cell lines. The Notch-1 sites in the promotor of GATA-3 at -3523 and the sites in exon 2 are occupied by Notch-1 proteins in each the cell lines tested (Fig. 6 and S3). Inhibition of NFκB activity or downregulation of Notch-1 expression showed that this binding is functionally relevant, as inhibiting these transcription factors caused a marked downregulation of GATA-3. Thus, the deregulated, constitutive activity of NFκB and Notch-1 in HRS cells contributes to the aberrant expression of GATA-3. Activating Notch-1 mutations in HL cell lines as a cause for its constitutive activity can be ruled out, since a mutational analysis of five HL cell lines did not reveal any activating mutations (data not shown). A picture is hence emerging in which multiple transcription factors influence each other and together cause the "reprogramming" of the HRS cells and the hyperactivated phenotype of these cells that is rather unique among lymphoid
malignancies. It remains to be determined which are the initial factors that cause the deregulation of numerous signalling pathways and transcription factors, and whether there are master regulators that stabilize the typical HRS cell gene expression pattern.

Taken together, we showed here that GATA-3 influences multiple cytokines and activators (herein IL-5, IL-13, and STAT-4) in cHL. By contributing to the regulation of these and additional factors, GATA-3 influences gene expression in HRS cells and their interaction with other cells in the microenvironment, and hence likely shapes the tumor physiology. Furthermore, we could show that the constitutively active transcription factors NFκB and Notch-1 contribute to the deregulated GATA-3 expression, which itself activates the expression of the STAT-4 transcription factor, pointing to a multi-component, interrelated and deregulated transcription factor network in HRS cells.

Acknowledgements

We thank Andrea Wind and Katharina Waldhelm for excellent technical assistance, Klaus Lennartz for support during FACS sorting, and Gwen Lorenz and Roland Schmitz for help with Notch-1 mutation analysis. We are indebted to Ralf Lieberz and Sylvia Hartmann for their expertise and performance of immunohistochemical stainings. This study was supported by grant KU 1315/5-2 from the Deutsche Forschungsgemeinschaft.
Authorship Contributions

J.S. designed and performed research, analyzed data and wrote the manuscript. C.D. performed statistical analysis. R.K. designed and supervised the research and wrote the manuscript. M.-L.H. provided biopsy material, and supervised and evaluated the immunohistochemical stainings.

Disclosure of Conflicts of Interest

The authors declare no competing financial interests.
References


Figure legends

**Figure 1. GATA-3 expression in cHL cell lines is effectively altered by shRNA knockdown**

(A) Immunoblots of HL cell lines L-428, L-591, L-1236, KM-H2, HDLM-2, U-HO1, and DEV. Same amount of protein (100 μg) were assayed for protein expression of GATA-3. Jurkat T cells were used as positive control. Actin was used as loading control to demonstrate equal loading. (B) Protein lysates of cell lines treated with lentiviral particles for two functional shRNAs (siGATA-3 #1 & #2, lanes 3 and 4) and one control scrambled shRNA (siSCR, lane 2) for GATA-3 were immunoblotted for GATA-3 and actin expression. Lysates were prepared three days (L-1236), four days (U-HO1), or five days post infection (L-428, KM-H2). (C) Three days post infection same numbers of GFP-positive cells were sorted, and after RNA and cDNA preparation, semi-quantitative PCRs for GATA-3 and GAPDH mRNAs were performed. In the No RT control RNA of untreated cells was deposited into the cDNA reaction without RT enzyme. One representative experiment of at least three independent repeated experiments is shown.

**Figure 2. GATA-3 downstream targets IL-5, IL-13, and STAT-4 are influenced by GATA-3 downregulation**

Semi-quantitative RT-PCR on sorted GFP-positive cells as in Fig. 1C on KM-H2 (A), L-428 (B), U-HO1 (C), and L-1236 (D) cells. mRNAs of IL-5, IL-13, and STAT-4 were amplified. A representative experiment of three independent experiments is shown for each cell line.

**Figure 3. GATA-3 and IL-13 expression correlate in primary cHL tissue**

Sixteen primary cases of cHL were analyzed for their correlation of GATA-3 and IL-13 following immunohistochemical staining. Samples 1, 5, 6, 8, 9 11-13, and 15 are of the NS
subtype. Samples 2-4, 7, 10, 14 and 16 are of the MC subtype. The Pearson correlation test resulted in a p-value of 0.0025. The T distribution hypothesis test gave a correlation coefficient of 0.619 (cor=1 for a perfect linear correlation). In this analysis, three cases with only cytoplasmic staining for GATA-3 were considered as "negative", because only nuclear GATA-3 can bind to the IL-13 promoter.

**Figure 4. GATA-3 regulates IL-13 expression in cHL cell lines**

(A) Cell culture supernatant of siSCR, siGATA-3#1, and siGATA-3#2 transduced L-1236 cells 3, 5, and 7 days post infection were analyzed for IL-13 amounts by ELISA in triplicates. Statistical significance was determined using a double-sided t test. (B) Protein lysates from cells corresponding to cell culture supernatants in (A) were prepared and 100 µg of protein were loaded onto SDS-PAGE. GATA-3 abundance was detected with antibody sc-268. Actin served as loading control. One representative experiment of three independent experiments is shown.

**Figure 5. GATA-3 has no anti-apoptotic or pro-survival effect in HL**

(A) MTT assay on transduced L-1236 cells 2, 3, 5, and 7 days post infection (dpi). Results for mock infected cells were set as 100% viable cells. The values are the mean of triplicate measurements. (B) Protein lysates of L-1236 cells following viral shRNA transfer were assayed for GATA-3, cleaved caspase-3, and actin abundance 5 days post infection. Treatment with doxorubicin for 48 h served as a positive control for apoptosis induction. One representative assay of three independent experiments is given.

**Figure 6. GATA-3 promoter and regulatory element sequences are occupied by NFκB subunit p65 and Notch-1 in vivo**
(A) Schematic draft of the GATA-3 gene. Exons 1 and 2 are depicted. The transcriptional start site (Txn) is at -953. The translational start site (Tln) is located in exon 2 at position +1. Exon 2 starts at -369. NFκB sites are indicated by white hexagons whereas black hexagons represent Notch-1/CSL sites. The three PCR amplicons are depicted by bars underlining putative binding sites. The arrows represent the primers used for PCR. (B) Results of a representative ChIP PCR following antibody treatment of samples in the indicated lanes for putative Notch-1 binding sites. (C) As in (B) PCRs for NFκB ChIP. Each ChIP was repeated twice.

Figure 7. NFκB activity and Notch-1 expression regulate GATA-3 expression

(A) Taqman PCR of one typical result of L-428, L-1236, KM-H2, and U-HO1 cells 5 days post viral transduction with IkBα superrepressor, GFP-positive cell sorting, and RNA/cDNA preparation. The three typical NKκB target Birc3, ICAM-1, and Lta were used as controls for NFκB activity. mRNA expression for all four analyzed genes is depicted as fold change downregulation after normalization to actin housekeeping controls. (B) Immunoblot for Notch-1 expression of HDLM-2, KM-H2, L-428, L-1236, and U-HO1 cells. Jurkat cells served as positive control. 100 μg protein were loaded. Actin was used as a loading control. (C) Taqman RT-PCR as in (A) on KM-H2, L-428, and U-HO1 cells 5 days post shRNA delivery against Notch-1. (D) Immunoblot for Notch-1 and GATA-3 expression in L-428 and U-HO1 cells 5 days following transduction of indicated shRNAs. Actin served as loading control. Each lentiviral transduction was performed three times.
Figure 1

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Figure 3

Percentage positivity of GATA3 and IL13

- GATA3
- IL13

p-value = 0.049

cor = 0.665
Figure 4

A

ELISA

amount of IL-13 [pg/ml]

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IB

GATA-3

Actin

3 days post inf. 5 days post inf. 7 days post inf.
Figure 5

A

MTT viability / %

Mock
siSCR
siGATA-3 #2

2dpi 3dpi 5dpi 7dpi
days post infection

B

IB

GATA-3

clv. caspase-3

Actin

19kD 17kD

5 days pi

17kD
Figure 6
Figure 7

A

Fold change downregulation

GATA-3 | BIRC3 | ICAM1 | LTA | GATA-3 | BIRC3 | ICAM1 | LTA | GATA-3 | BIRC3 | ICAM1 | LTA
L-428 | L-1236 | KM-H2 | U-H01

B

Jurkat | HDLM-2 | KM-H2 | L-428 | L-1236 | U-H01
Notch-1
Actin
Figure 7

C

Fold change downregulation

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Mechanisms of aberrant GATA-3 expression in classical Hodgkin lymphoma and its consequences for the cytokine profile of Hodgkin and Reed/Sternberg cells

Jens Stanelle, Claudia Döring, Martin-Leo Hansmann and Ralf Küppers