Hodgkin’s lymphoma associated T-cells exhibit a transcription factor profile consistent with distinct lymphoid compartments

Çigdem Atayar, Anke van den Berg, Tjasso Blokzijl, Marcel Boot, Randy D Gascoyne, Lydia Visser, Sibrand Poppema

Background: Hodgkin’s lymphoma (HL) is characterised by an ineffective immune response that is predominantly mediated by CD4+ T-cells.

Aims: To analyse the expression of the key regulatory T-cell transcription factors (TFs) in the T-cells of HL involved tissues in order to assess the nature of the TH immune response in HL.

Methods and results: By immunohistochemistry, GATA3 was strongly and T-bet exclusively expressed in a subset of interfollicular lymphocytes in the reactive lymphoid tissues. In classical HL (CHL), which is generally located in the interfollicular zones, a predominance of T-bet+ T-cells and lesser amounts of GATA3+ and c-Maf+ T-cells was found, concordant with the pattern of the normal interfollicular compartment. In reactive lymphoid tissues, c-Maf was observed mostly in T-lymphocytes within the germinal centres (GCs). Nodular lymphocyte predominance type of Hodgkin’s lymphoma (NLPHL) and progressively transformed germinal centres cases, showed a majority of c-Maf+ T-cells, consistent with the pattern in normal GCs. NLPHL cases uniformly showed c-Maf+/CD57+ T-cell rosettes around the neoplastic cells; these rosettes were absent in “paragranuloma-type” T-cell/histiocyte rich B-cell lymphoma.

Conclusions: T-cell TF expression profiles of the reactive T-cells in both subtypes of HL are in accordance with the expression profile observed in the distinct lymphoid compartments.

Abbreviations: CHL, classical Hodgkin’s lymphoma; GC, germinal centre; HL, Hodgkin’s lymphoma; HRBCL, histiocytoid rich B-cell lymphoma; HRS, Hodgkin and Reed–Sternberg; L&H, lymphocytic and/or histiocytic; HL, Hodgkin's lymphoma; L&H, lymphocytic and/or histiocytic; PTGC, progressively transformed germinal centre; TF, transcription factor; TTH, T-helper

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Table 1  Antibodies used in the present study

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-bet</td>
<td>4810 (monoclonal, IgG1)</td>
<td>Prof LH Glomcher, Harvard Medical School, Boston, MA, USA</td>
<td>1/50</td>
</tr>
<tr>
<td></td>
<td>52-3567 (polyclonal)</td>
<td>Zymed Laboratories Inc., San Francisco, CA, USA</td>
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<td>HG3-31 (monoclonal, IgG1)</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA, USA</td>
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<td>Santa Cruz Biotechnology, Santa Cruz, CA, USA</td>
<td>1/50</td>
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<tr>
<td>CD3</td>
<td>PS1 (monoclonal, IgG2a)</td>
<td>Ventana Medical Systems Inc., Oro Valley, AZ, USA</td>
<td>pre-diluted</td>
</tr>
<tr>
<td>CD57</td>
<td>PK1 (monoclonal, IgM)</td>
<td>Monosan, Uden, The Netherlands</td>
<td>1/40</td>
</tr>
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MATERIALS AND METHODS

Patients and tissue samples

Formalin-fixed, paraffin-embedded and frozen samples of a series of previously diagnosed HL tissues, comprising 16 CHL (7 female, 9 male; age 10–94 years, mean 40 years, median 33 years), 7 NLPHL (2 female, 5 male; age 17–73 years, mean 36 years, median 34 years), and 4 PTGC (2 female, 2 male; age 24–54 years, mean 33 years, median 26 years) cases were obtained from the files of the Department of Pathology & Laboratory Medicine, University Medical Centre Groningen, The Netherlands, according to institutional guidelines. The CHL group comprised 10 cases of nodular sclerosis type, 3 cases of mixed cellularity type, 1 case of lymphocyte-depleted type, and 2 cases of unclassifiable HL. All NLPHL cases showed a predominant nodular pattern together with minor diffuse areas involving usually less than 10–20% of the involved lymph node area. Two cases of PTGC had a HL history and 2 cases were de novo cases. In the PTGC cases, 10–25% of the follicles per tissue section were involved. We also studied 6 cases of “paragranuloma type” T/HRBCL that were previously described by Rudiger et al.14 Tissue blocks of 3 reactive tonsils, 5 reactive lymph nodes and 3 normal adult thymuses were used as control tissues. All samples were anonymised; full ethical approval in accordance with local policy was obtained by the institution’s review board for human subject research for obtaining and using these tissue samples.

Immunohistochemistry

Paraffin-embedded tissue sections (4 µm thick) were cut, mounted on aminopropyltriethoxysilane-coated slides (Lo-Laboroptik GmbH, Friedrichsdorf, Germany), dried, deparaffinised in xylene and rehydrated in graded alcohols. Antigen retrieval was carried out in a microwave oven (Amana, Louis, MO, USA) staining. For GATA3 staining 4 mm-thick sections of 3 reactive tonsils and lymph nodes were air-dried, fixed in acetone, and incubated for 1 hour with primary antibody, followed by application of HRP labelled rabbit-anti-mouse Ig and goat-anti-rabbit Ig (DAKO) incubation steps for 30 min each. 3-Amino-9-ethylcarbazole (Sigma–Aldrich) was used to visualise the positive cells. Positive control tissues as well as negative controls, sections stained in parallel without primary antibody, were used in all instances. The pattern and intensity (+, weak; ++, moderate; or ++++, strong) of the staining was also recorded. PTGC findings reported are the findings observed in the transformed follicles of PTGC cases. Reactive follicles and paracortical areas surrounding the transformed follicles had the same staining pattern as reactive/normal lymphoid tissues.

Double immunostaining

Double immunostaining was carried out on paraffin sections of reactive tonsils to confirm that T-bet and c-Maf was expressed by T-cells. To detect T-bet and c-Maf, the above mentioned protocol was followed; subsequently the same protocol was repeated using CD3 and CD57 (table 1) which was developed with rabbit-anti-mouse Ig and goat-anti-rabbit Ig conjugated to alkaline phosphatase (DAKO) and Fast Blue. No counter stain was used. Single staining for T-bet, c-Maf, CD3, and CD57 was also performed by applying all the other steps used for the double staining to exclude cross-reaction of the different antibodies. Negative control staining (incubation without primary antibody) was performed routinely.

RESULTS

Strong expression of T-bet in reactive infiltrate of CHL cases

In thymic cortex and medulla only a few cells exhibited moderate to strong T-bet positivity (fig 1A, table 2). In GGs of tonsil or reactive lymph node, virtually no T-bet staining was observed (fig 1B). Staining was restricted to the interfollicular compartments as well as the subepithelial area in the tonsil. In reactive lymph nodes, interfollicular lymphocytes as well as marginal sinus lymphocytes stained intensely for T-bet and were especially prominent in vascular and perivascular areas. In addition to the mature lymphocytes, some macrophages also showed reactivity with T-bet antibody in moderate intensity. In PTGC cases strongly stained cells were present in the interfollicular area similar to reactive lymph nodes. In two cases T-bet expression was observed in less than 10% of the non-neoplastic lymphocytes which were preferentially located in perivascular areas. One case in which we observed strong T-bet immunoreactivity in 25–50% of L&H cells also exhibited intense T-bet reactivity in reactive lymphocytes. In 14 CHL cases the majority of T-lymphocytes stained intensely for T-bet (fig 1C).

NLPHL cases showed weak staining (fig 1D) to moderate staining in 5–10% of the non-neoplastic lymphocytes which were preferentially located in perivascular areas. One case in which we observed strong T-bet immunoreactivity in 25–50% of L&H cells also exhibited intense T-bet reactivity in reactive lymphocytes. In 14 CHL cases the majority of T-lymphocytes showed moderate to strong staining (fig 1E) T-bet reactivity (table 3).

In two cases T-bet expression was observed in less than 10% of the T-lymphocytes. Remarkably, the HRS cells of these two cases were also negative for T-bet staining, whereas in the rest of the cases HRS cells also expressed T-bet. Double immunostaining with CD3 on tonsil sections revealed that ~20% of the CD3 T-cells were T-bet+. These T-bet+ cells were exclusively located in the interfollicular area. On the other hand, ~50% of the T-bet+ cells were expressing CD3 in tonsil. On CHL sections...
(fig 2A), 80–90% of the CD3+ cells were also T-bet+ and more than 90% of the T-bet+ cells were CD3+.

Low levels of GATA3 expression in T-cell infiltrates of HL

In thymus most cortical thymocytes had cytoplasmic GATA3 and only some had nuclear GATA3, while medullary lymphocytes had exclusively nuclear GATA3 staining (fig 1F). The staining in thymus was generally moderate to strong in intensity. In tonsil and reactive lymph nodes strong nuclear GATA3 immunoreactivity was seen in scattered extrafollicular lymphocytes (table 2). Eosinophils, with strong cytoplasmic GATA3 staining, were also detected in interfollicular areas. In the light and dark zones of GCs a staining of weak to moderate intensity was observed, especially in larger lymphocytes. In the mantle zone some lymphocytes showed weak GATA3 immunoreactivity (fig 1G). In PTGC both transformed follicles and enlarged mantle zone displayed moderately or weakly interspersed positive cells, whereas in interfollicular areas strongly GATA3+ lymphocytes were observed similar to the reactive lymph node (fig 1H).
GATA3 immunostaining was observed at very low numbers of NLPHL associated T-cells (fig 1J). The positive T-lymphocytes within the nodules were distributed randomly with no spatial relation to tumour cells. In most CHL cases 10–25% of background lymphocytes were GATA3+ (table 3). In two cases, 25–50% of the reactive infiltrate showed a moderate staining intensity. When we performed double immunostaining on tonsil sections with CD3, 90–95% of the c-Maf+ cells were CD3+. These cells were predominantly located in GCs. Moreover, 5–10% of the CD3+ T-cells were c-Maf co-positive. Double immunostaining with CD57 on tonsil sections revealed that 60–70% of the c-Maf+ cells were co-expressing CD57 and these cells were exclusively located within the GCs. In total more than 90% of the CD57+ T-cells were c-Maf co-positive. In the interfollicular areas, intensely stained cells were interspersed. Strongly c-Maf+ cells were highly increased in numbers in transformed follicles of PTGC cases (fig 1M). In contrast to normal GC, there was no regional distribution of c-Maf+ cells in PTGC. Although few in number, some c-Maf+ collarettes with strong staining intensity were observed around large lymphocytes in PTGC (fig 1M, inset). These scattered rosette like formations were found especially in the enlarged mantle zone. Single cells that were c-Maf+ were also observed in the expanded mantle zone.

Striking expression of c-Maf in NLPHL and PTGC, but not in “paragranuloma type” T/HRBCL

In thymus, scattered larger thymocytes in the cortex stained strongly with c-Maf, whereas in the medulla large cells as well as interspersed smaller cells were stained with weak to moderate intensity (fig 1K, table 2). c-Maf+ T-cells were predominantly detected in the light zone of GCs and just beneath the follicular mantle, even though they were also observed in the other compartments including the mantle zone and interfollicular area (fig 1L). Some of the GC macrophages were also stained in moderate intensity. When we performed double immunostaining on tonsil sections with CD3, 90–95% of the c-Maf+ cells were CD3+. These cells were predominantly located in GCs. Moreover, 5–10% of the CD3+ T-cells were c-Maf co-positive. Double immunostaining with CD57 on tonsil sections revealed that 60–70% of the c-Maf+ cells were co-expressing CD57 and these cells were exclusively located within the GCs. In total more than 90% of the CD57+ T-cells were c-Maf co-positive. In the interfollicular areas, intensely stained cells were interspersed. Strongly c-Maf+ cells were highly increased in numbers in transformed follicles of PTGC cases (fig 1M). In contrast to normal GC, there was no regional distribution of c-Maf+ cells in PTGC. Although few in number, some c-Maf+ collarettes with strong staining intensity were observed around large lymphocytes in PTGC (fig 1M, inset). These scattered rosette like formations were found especially in the enlarged mantle zone. Single cells that were c-Maf+ were also observed in the expanded mantle zone.
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NLPHL. c-Maf
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T-bet
commitment, in the reactive cells of HL.

T-cells were c-Maf
histological similarity to NLPHL. In general, about 15–20% of
in the vicinity of the HRS cells were also observed, but there
distributed in the NLPHL nodule. Original magnification ×157.5.

In the majority of NLPHL cases, 25–50% of the lymphocytes
showed moderate to strong expression of c-Maf (table 3). High
numbers of positive cells with a strong staining intensity for
c-Maf were detected preferentially within the neoplastic follicles
and in the lymphocytes, forming rosettes around the L&H cells
(fig 1N and inset). A lower number of c-Maf+ cells were observed outside of the nodules of NLPHL. While strongly
expressed on rosetting cells, a weak to moderate staining
intensity was observed on non-rosetting lymphocytes. Double
staining of NLPHL sections with c-Maf and CD57 revealed the
abundant presence (90–95%) of the c-Maf+ cells were CD57+
and almost all CD57+ cells were c-Maf+ of c-Maf+/CD57+ phenotype
T-cells as rosette formations around lymphocytic and/or histiocytic cells, or widely

distributed pattern. The presence of T-cells with a different T-cell
distribution pattern. The presence of T-cells of NLPHL have characteristics of GC B-cells, and the
cells of NLPHL have characteristics of GC B-cells, and the

DISCUSSION
In the present study, we analysed the expression pattern of
three T-cell TFs, considered to be important in T3 lineage
commitment, in the reactive cells of HL. T-bet17 and GATA318 19
were identified as master factors regulating induction of T31 and T32 pathways respectively, c-Maf is a basic leucine zipper
transcription factor that is expressed in T32 cells and has a
selective function in regulation of interleukin 4 (IL4) transcrip-
tion.20 c-Maf expression has also been reported in multiple
myeloma,21 and T-bet expression has been found in normal B-
cells22 and some B-cell neoplasms.13 14 To show the T-cell nature of the positive cells we performed double immunostainings
with T-cell specific markers on control tonsil sections. These
revealed that the vast majority of the c-Maf+ and T-bet+ cells
were indeed of T-cell origin.

We have shown that the reactive T-cells in CHL cases are
predominantly immunoreactive for T-bet. The T-cells in CHL
include cells expressing IL2, IL10, IL13, IFNγ, TGFB1,2 and
FOXP33 and can be regarded as having a T32-like or immuno-
regulatory phenotype.4 5 This cytokine profile is not consistent
with the “master T31 transcription regulator”17—T-bet expression
in the reactive T-cells of CHL. However, T-bet expression is not
necessarily incompatible with T32 cytokine production since
retroviral expression of T-bet into T32 cells does not extinguish
expression of GATA3 or T32 cytokines.24 Moreover, T-bet
immunoreactivity in the reactive T-cells of CHL does not indicate
a T31 type immune response per se; recently a new type of CD4+/CD25+
adaptive T-regulatory cell has been identified that is
phenotypically related to T31 cells with expression of T-bet,
FOXP3, ICOS, IL10 and IFNγ.25 Raised levels of FOXP3+ T-cells
were indeed described in CHL, albeit in a low percentage of cases.26
Therefore, it is possible that the TF expression repertoire of the
reactive T-lymphocytes of CHL indicates a specific subpopulation
of T-regulatory cells.

In reactive lymphoid tissues, T-bet27 as well as FOXP33 T-cell
populations were observed solely in the interfollicular compart-
ment of the reactive lymphoid tissues; this is consistent with
the presence of CHL in the interfollicular compartment of the
lymph nodes.

In the CHL background population, we also detected GATA3+ and c-Maf+ cells at low numbers which did not exhibit a specific
distribution pattern. The presence of T-cells with a different T-cell
TF expression repertoire suggests various subpopulations with
different specificities and functions. On the other hand, during the
T-cell differentiation process, before achieving restricted expres-
sion in the fully polarised cell, persistent co-expression of both
types of T-cell TFs has been reported in both T31 subsets.28
Therefore, it is also plausible that part of the reactive T-cells of
HL are not yet fully committed or have retained the ability to
activate and express T31 and T32 type TFs together.

We have shown that c-Maf+ T-cells are predominantly
located within the GCs of the reactive lymphoid tissues. L&H
and the nodules of NLPHL are considered altered germinal centres.27
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activate and express T31 and T32 type TFs together.

We have shown that c-Maf+ T-cells are predominantly
located within the GCs of the reactive lymphoid tissues. L&H
cells of NLPHL have characteristic features of GC B-cells, and
the nodules of NLPHL are considered altered germinal centres.27
Thus, L&H cells, surrounded by c-Maf− T-cells, proliferate in a
GC-like environment. The CD57− T-cell rosetting pattern is a
distinct and well known feature of NLPHL,1 however this is the first
demonstration of c-Maf+/CD57− T-cell rosettes around L&H
cells. Carbone et al28 showed the presence of MUM1/IRF4+ T-
cells, which are located mainly in close proximity to L&H cells
as rosettes. c-Maf is a specific TF for IL4,20 and MUM1/IRF4
synergises with NFATc2 and c-Maf, to augment IL4 promoter
activity as well as to elicit significant levels of endogenous IL4
production.29 However, we have recently reported that in
contrast to their tonsillar counterparts, CD4+/CD57− T-cells of
NLPHL do not express IL4 even after PMA–ionomycin
activation.2 This suggests that in NLPHL, c-Maf and MUM1/
IRF4 induced expression of IL4 is blocked. c-Maf also has a
transforming ability,30 and has been shown to function as an
oncogene.31 The functional significance of c-Maf+ T-cell rosettes
in NLPHL cases remains to be elucidated.

Figure 2 Double immunostaining of Hodgkin’s lymphoma sections with T-
cell markers. (A) Double staining for T-bet and CD3 in classical Hodgkin’s
lymphoma (CHL) showed that the vast majority of the T-bet expressing
background cells of CHL were CD3− T-cells. Hodgkin and Reed–Sternberg
cells were also strongly T-bet+.

(B) Double staining of nodular lymphocyte
predominance type of Hodgkin’s lymphoma (NLPHL) with c-Maf and CD57
revealed the abundant presence of c-Maf+/CD57− phenotype T-cells as
rosette formations around lymphocytic and/or histiocytic cells, or widely
distributed in the NLPHL nodule. Original magnification ×157.5.

Figure 3 Paragranuloma type T/HRBCL. (A) H&E staining of
“paragranuloma” type T/HRBCL with neoplastic cells resembling
“popcorn” cells of NLPHL and the surrounding non-neoplastic
lymphocytes. (B) c-Maf staining of paragranuloma type T/HRBCL showed
that the c-Maf+ cells were less in number (in this case 20%) compared to
NLPHL c-Maf+ cells were randomly distributed and no c-Maf+ rosettes were
present. Original magnification ×157.5.
T-cell transcription factors in Hodgkin’s lymphoma

NLPHL can histologically and immunophenotypically resemble a subset of T/HDRBCL cases.\(^2\) To investigate whether the presence of c-Maf\(^+\) rosettes is of value in the differential diagnosis between T/HDRBCL and NLPHL, we have stained a specific subtype of cases referred to as “paragranuloma type” of T/HDRBCL.\(^3\) for c-Maf antibody. None of the six paragranuloma type of T/HDRBCL cases showed c-Maf\(^+\) rosettes but rather showed a “CHL pattern” of c-Ma staining. Therefore, the presence of c-Maf\(^+\) rosettes may help to distinguish NLPHL from paragranuloma type of T/HDRBCL. In fact, three immunophenotypic features can be used to distinguish NLPHL from T/HDRBCL, including two markers of the neoplastic B cells (LSP-1, PU.1)\(^3\) and one differently expressed by the non-neoplastic T cells (c-Maf).

PTGC have been consistently been associated with NLPHL.\(^1\)\(^3\)\(^9\) Our results further support the hypothesis that PTGC and NLPHL may both be manifestations of an abnormal follicular centre reaction. c-Maf staining was most pronounced in the light zone of GC of the reactive lymph nodes (tables 2 and 3). In transformed follicles of PTGC cases there was an increased frequency of c-Maf\(^+\) T-cells together with loss of zonal distribution of c-Maf reactivity. Frequent c-Maf\(^+\) T-cell rosettes around L&H cells was a common characteristic of all NLPHL cases, and c-Maf\(^+\) rosette like formations were also detected between broken-up borders of GC and the mantle zone in transformed follicles of PTGC, albeit at low frequencies. PTGC and NLPHL nodules contained very few GATA3\(^+\) and T-bet\(^+\) T-lymphocytes, which is consistent with an enlarged mantle zone like B-cell rich area.

In conclusion, we have shown that CHL associated lymphocytes are mostly T-bet expressing T-cells, whereas NLPHL cases have a high frequency of c-Maf\(^+\)/CD57\(^+\) T-cells as rosettes or single cells. The T-cell TF expression pattern of the HL associated T-cells is consistent with the T-cell TF pattern observed in the distinct lymphoid compartments in which HL subtypes are diagnosed. This implies that background T-cells of HL are not randomly distributed T-cells, but an expansion of the native T-cells or an influx of T-cells with the same profile as the native T-cells in the corresponding lymphoid compartments.

Take-home messages

- Background T-cells of Hodgkin’s lymphoma (HL) are not randomly distributed T-cells: the reactive T-cells in classical Hodgkin’s lymphoma (CHL) are predominantly immunoreactive for T-bet and in NLPHL for c-Maf.
- T-cell transcription factor profiles of HL indicate that the two types of HL are two different diseases within two distinct lymphoid compartments.
- Raised numbers of c-Maf\(^+\)/CD57\(^+\) T-cell rosettes are characteristic for NLPHL.

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