Deep sequencing of bone marrow microenvironments of patients with del(5q) myelodysplastic syndrome reveals imprints of antigenic selection as well as generation of novel T-cell clusters as a response pattern to lenalidomide

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ABSTRACT

In myelodysplastic syndromes with a partial deletion of the long arm of chromosome 5, del(5q), lenalidomide is believed to reverse anergic T-cell immunity in the bone marrow resulting in suppression of the del(5q) clone. In this study we used next-generation sequencing of immunoglobulin heavy chain (*IGH*) and T-cell receptor beta (*TRB*) rearrangements in bone marrow-residing and peripheral blood-circulating lymphocytes of patients with del(5q) myelodysplastic syndromes to assess the immune architecture and track adaptive immune responses during treatment with lenalidomide. The baseline bone marrow B-cell space in patients was comparable to that of age-matched healthy controls in terms of gene usage and *IGH* clonality, but showed a higher percentage of hypermutated *IGH* sequences, indicating an expanded number of antigen-experienced B lineage cells. Bone marrow B lineage clonality decreased significantly and hypermutated *IGH* clones normalized upon lenalidomide treatment, well in line with the proliferative effect on healthy antigen-inexperienced B-cell precursors previously described for this drug. The T-cell space in bone marrow of patients with del(5q) myelodysplastic syndromes showed higher *TRB* clonality compared to that of healthy controls. Upon lenalidomide treatment, myelodysplastic syndrome-specific T-cell clusters with low to medium spontaneous generation probabilities emerged; these clusters were shared across patients, indicating a common antigen-driven T-cell response pattern. Hence, we observed B lineage diversification and generation of new, antigen-dependent T-cell clusters, compatible with a model of adaptive immunity induced against the del(5q) clone by lenalidomide. Overall, this supports the concept that lenalidomide not only alters the functional T-cell state, but also the composition of the T- and B-cell repertoires in del(5q) myelodysplastic syndromes.

Introduction

Myelodysplastic syndromes (MDS) are clonal stem cell disorders characterized by inefficient hematopoiesis. The most prevalent cytogenetic abnormality in these diseases is a partial deletion of the long arm of chromosome 5, del(5q), which is present in about 15% of patients.[1] This clinically distinct entity is char-

acterized by refractory anemia, often thrombocythemia with atypical megakaryocytes in the bone marrow (BM) and a relatively low progression rate to acute myeloid leukemia compared to the rate of transformation of other types of MDS.[2] The pathogenesis seems to be partially driven by haploinsufficiency of genes located in the deleted area on chromosome 5.[3-8] Moreover, there is evidence of adaptive and innate immune dysregulation with altered cytokine networks, diminished NK cell activity and an anergic T-cell environment failing to suppress the defective del(5q) clone.[9]

In 2005, lenalidomide was approved in the USA for the treatment of lower-risk, transfusion-dependent del(5q) MDS patients as the first immunomodulatory drug. In the placebo-controlled phase III trial MDS-004, 56% of patients who received 10 mg/day lenalidomide achieved red blood cell transfusion independency for more than 26 weeks.[10] Response to lenalidomide was shown to be associated with significant improvement in survival with a median overall survival of 4 years.[11] Cereblon (CRBN), a substrate receptor of the CRL4 ubiquitin ligase complex, is the primary molecular target through which lenalidomide mediates its anti-tumor effects. Krönke *et al.* demonstrated that lenalidomide induces the ubiquitination and degradation of IKZF1, IKZF3 and CK1α by binding to CRBN.[12,13] CK1α, a negative regulator of p53, may be of particular pathophysiological relevance in the context of MDS del(5q), since it is encoded by a gene within the deleted chromosomal region and haploinsufficient expression sensitizes cells to lenalidomide therapy.[12] Furthermore, the ubiquitin-independent physiological chaperone-like function of CRBN is targeted by lenalidomide. Eichner *et al.* found that lenalidomide outcompetes CRBN for binding to and stabilizing the CD147-MCT1 complex.[14] This complex promotes various biological functions, including angiogenesis, proliferation and invasion. Since recent evidence suggests a negative regulatory role of CD147 in T-cell-mediated immune responses,[15-17] lenalidomide-induced CD147 destabilization has been speculated to explain not only direct anti-tumor effects, but also immune cell activation.[14] Functionally, the immunomodulation induced by lenalidomide was described as a reversal of pathological T-cell tolerance by increasing T-cell effector function and secretion of interleukin-2, interferon-γ and tumor necrosis factor-α by CD4+ and CD8+ T cells.[18] Expanding on this, a recent RNA sequencing study on non-del(5q) MDS patients suggested that the immunomodulatory signal delivered by lenalidomide may also affect the composition rather than only the functional state of the local BM immune cells.[19] However, the immunomodulatory effects of lenalidomide on the microenvironment have not yet been fully defined.

In this study, we analyzed local BM and peripheral blood (PB) environments to understand the clonal immune architecture of MDS del(5q) and its modulation by lenalidomide. To do this, we used state-of-the-art next-generation sequencing (NGS) of immunoglobulin heavy chain (*IGH*) and T-cell receptor beta (*TRB*) rearrangements as an advanced technology that enables simultaneous identification of tens of thousands of unique B-cell and T-cell receptor rearrangements from a single tissue sample as well as tracking over time. These analyses demonstrated that lenalidomide shapes T-cell adaptive immune responses in the BM niche of patients with MDS del(5q).

Methods

Patients' characteristics

Patients' BM samples were collected between 2013 and 2016 at baseline and after a median of 12 months of lenalidomide treatment in the course of the LE-MON 5 and Bioregister trials. The cohort comprised 15 patients with del(5q) MDS. Matching pre- and post-treatment PB samples were available for eight of these patients. All del(5q) patients were treated in the LE-MON 5 trial,[20] a German multicenter, single-arm, open-label, phase II study [Safety of Lenalidomide Monotherapy and Markers for Disease Progression in Patients With IPSS Low- or Intermediate-1 Risk Myelodysplastic Syndromes (MDS) Associated With an Isolated Deletion 5q Cytogenetic Abnormality] according to the standard lenalidomide protocol.[10] As a reference, seven BM and 22 PB samples from age-matched healthy controls without hematologic abnormalities were included. Five BM samples were collected during hip replacement surgery and two were obtained from iliac crest aspiration of healthy individuals, without apparent differences. Hemograms of all the healthy donors were normal as were their C-reactive protein levels. The BM of all MDS patients was obtained by standardized aspiration and biopsy of the iliac crest. All MDS del(5q) samples analyzed in this study were previously subjected to NGS of TP53,[21] and were negative for TP53 mutations. The patients' characteristics are shown in Table 1.

Study approval

Informed consent was obtained from all patients and healthy controls for the use of their diagnostic material for scientific purposes approved by the institutional review board (Ethikkommission der Medizinischen Fakultät, Henrich Heine University Düsseldorf, Germany, project number MC-LKP-392).

Isolation of genomic DNA

Genomic DNA was isolated from >10^6 frozen BM or PB mononuclear cells using a Gen Elute mammalian genomic DNA miniprep kit (Sigma-Aldrich, Taufkirchen, Germany) according to the supplier's suggestions.

Multiplex polymerase chain reaction analysis for *IGH* and *TRB* repertoire amplification for Illumina targeted next-generation sequencing

Both the *IGH* and *TRB* genes containing the entire rearranged *V, D* and *J* segments were amplified with BIOMED2-FR1 (*IGH*), -TRB-A and -B primer pools starting from 250 ng of input genomic DNA.[22] Amplicons were tagged with Illumina adapters and indices in two consecutive polymerase chain reactions (PCR), as previously described.[23] Briefly, previously published primers[22-24] for the first PCR annealed within either the *IGH* or *TRB* loci and contained Illumina-compatible adapters for NGS primer annealing. A second PCR was performed using outer primers to extend the Illumina adapter for later hybridization of amplicons to the Illumina flow cell and to add a seven nucleotide barcode, allowing each sequence to be matched during data analysis to a certain patient and time point. All primers were purchased from Metabion (Martinsried, Germany). All PCR were performed using Phusion HS II polymerase (Thermo Fisher Scientific Inc., Darmstadt, Germany). Amplicons with the expected size were purified after agarose gel electrophoresis using a NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). The concentration of the amplicons was determined on Qubit (QIAGEN, Hilden, Germany) and the quality of the amplicon pools was controlled on an Agilent 2100 Bioanalyzer (Agilent technologies, Böblingen, Germany) before being subjected to NGS.

Illumina next-generation sequencing and data analysis

NGS and demultiplexing were performed on an Illumina MiSeq sequencer (600-cycle single indexed, paired-end run, V3-chemistry). Analysis of rearranged *IGH* and *TRB* loci was computed using the MiXCR and ENPICOM's ImmunoGenomix Platforms analysis tools.[23,25] *In silico* grouping of lymphocyte interactions by paratope hotspots (GLIPH) cluster and generation probability analyses were performed as described in previous publications.[26,27]

Repertoire metrics analyses

Clonality is an index calculated according to the formula "1 - Pielou's evenness".[28] Pielou's evenness is calculated according to the formula $J = H'/\ln(S)$ where H' is the Shannon-Wiener diversity index and S is the total number of clones in a specific sample.[29] The clonality index ranges from 0 to 1, with 0 indicating complete diversity and 1 indicating absolute clonality of the investigated sample and thus the presence of only one clone.

In silico GLIPH and generation probability analyses

We applied the GLIPH algorithm to our NGS-generated healthy donor and MDS patient *TRB* dataset.[26] Next we investi-gated the generation probability of the GLIPH-generated *TRB* clusters of our healthy controls and MDS patients with the IGoR algorithm[27] and used the negative logarithmic value to display low probability values better. Clusters with low generation probability are expected to be generated by antigen selection, although no cutoff value defining "low" exists, to the best of our knowledge. For a better description of our data we decided to define cutoffs in our study by dividing the range between the lowest (13.1400259) and highest (36.7368006) observed generation probability values into three equally sized areas represent-ing high (a value in the range of 13.1400259-21.0056175), medi-um (21.0056176-28.871209) and low (28.871210-36.7368006) probability. T-cell clusters that expand after lenalidomide treat-ment and have a low generation probability are expected to have been generated by antigen selection, whereas clusters which expand but have a high generation probability are less likely to have expanded by antigen selection.

Statistics

Differences in distributions were studied using the Student *t*-test. All statistical analyses were performed with GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

Table 1. Characteristics of the cohort of patients with del(5q) myelodysplastic syndromes.

Patient n.	Age at first diagnosis	Sex	WHO diagnosis	IPSS	Samples	Months after 1st sample	Response at sample collection	Best response
1	66	F	Isolated del5q	Int-1	BM, PB	0 12	Baseline PR	PR/TI
2	75	F	Isolated del5q	Int-1	BM, PB	0 16	Baseline No treatment	No change/SD
3	78	F	Isolated del5q	Int-1	BM, PB	0 7	Baseline PR	TI
4	68	F	Isolated del5q	Int-1	BM	0 15	Baseline PD (morphology)	TI
5	74	M	Isolated del5q	Int-1	BM, PB	0 30	Baseline NA	CCR/TI
6	52	M	Isolated del5q	Int-1	BM, PB	0 7	Baseline SD	NA
7	71	M	Isolated del5q	Low	BM, PB	0 18	Baseline CR	CCR/TI
8	63	F	Isolated del5q	Int-1	BM, PB	0 6	Baseline SD	NA
9	76	F	Isolated del5q	Low	BM, PB	0 42	Baseline PR/CCR?	CCR/TI
10	48	F	Isolated del5q	Int-1	BM	0 30	Baseline Relapse	CCR/TI
11	68	F	Isolated del5q	Int-1	BM	0 12	Baseline PD (RAEB-2)	PR/TI
12	74	M	Isolated del5q	Int-1	BM	0 6	Baseline PD (RAEB-2)	TI
13	60	M	Isolated del5q	Int-1	BM	0 6	Baseline CR	CCR/TI
14	51	F	Isolated del5q	Int-1	BM	0 12	Baseline CR	CCR/TI
15	45	F	Isolated del5q	Int-1	BM	0 6	Baseline CR	CCR/TI

N: number; F: female; M: male; WHO: World Health Organization; Isolated del5q: myelodysplastic syndrome with isolated deletion of chromosome 5q; IPSS: International Prognostic Scoring System; Int-1: intermediate risk 1; BM: bone marrow; PB: peripheral blood; TI: transfusion independence; CR: complete remission; CCR: complete cytogenetic remission; PR: partial remission; SD: stable disease; PD: progressive disease; RAEB-2: refractory anemia with excess blasts-2; NA: not available; Baseline: indicates sample taken before the initiation of lenalidomide treatment..
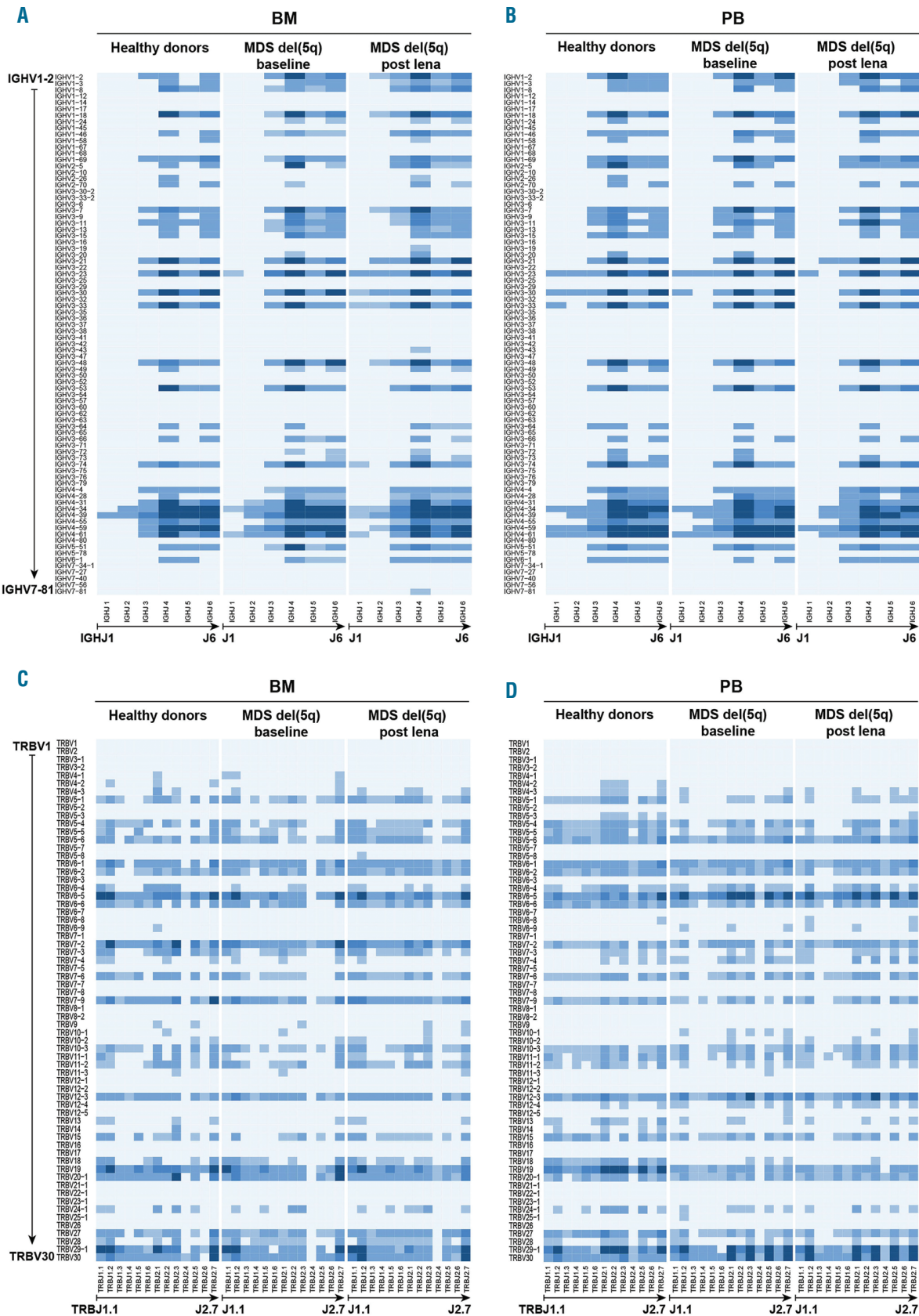
Figure 1. *TRB* and *IGH V*- and *J*-gene usage before and after lenalidomide treatment in patients with del(5q) myelodysplastic syndromes and in healthy controls. For each sample from healthy controls and patients with del(5q) myelodysplastic syndromes before (baseline) and after lenalidomide therapy, the frequency of the *V-J* gene combinations of all *IGH* and *TRB* clones was calculated separately as a percentage. The median frequencies, in percentages, are shown for (A,B) *IGH* and (C,D) *TRB V-J*-gene combinations for healthy controls and patients, before and after lenalidomide treatment, in (A,C) bone marrow and (B,D) peripheral blood samples as heat maps. Light blue represents lowest frequency and dark blue highest frequency. For both *IGH* and *TRB*, *J* genes are listed along the x axis and functional *V* genes along the y axis. BM: bone marrow; PB: peripheral blood; MDS; myelodysplastic syndrome; baseline: prior to lenalidomide treatment; post lena: after lenalidomide treatment

Results

No disease-specific gene usage or shared individual B- or T-cell clones in immune environments of patients with del(5q) myelodysplastic syndromes

In search of a repertoire signature of immune environments in del(5q) MDS or a signature of treatment response to lenalidomide, we globally compared B- and T-cell spaces of our cohort of del(5q) MDS patients (characteristics shown in Table 1) with those of healthy, age-matched controls. To this end, next-generation immunosequencing was performed on the T lineage *TRB* locus and the B lineage *IGH* locus in all subjects at baseline as well as 6-42 months after initiation of lenalidomide treatment. As regards *TRB* and *IGH* gene usage, del(5q) MDS samples did not differ from samples from age-matched healthy donors and no systematic biases in gene usage were observed upon lenalidomide treatment (Figure 1A-D). Moreover, no disease-specific shared B- or T-cell clones were discernable (Figure 2). Interestingly, PB and BM samples as well as follow-up samples from the same patient showed significant overlapping for *TRB* (Figure 2B), but not for *IGH* (Figure 2A).

The clonal bone marrow B-cell space diversifies upon lenalidomide treatment in del(5q) myelodysplastic syndromes

Next, we explored B-cell architectural changes upon lenalidomide treatment by comparing pre- and post-lenalidomide *IGH* repertoires. The pre-lenalidomide BM B-cell space in del(5q) MDS was comparable to the B-cell space of age-matched healthy donors in terms of *IGH* clonality, but showed a significantly higher somatic hypermutation rate of *IGH* clones, indicating an extended number of antigen-experienced B lineage cells (Figure 3A,C). BM (but not PB) B lineage clonality decreased significantly even below the levels in the age-matched healthy donor group and somatic hypermutation rates of *IGH* clones normalized in BM and PB upon lenalidomide treatment (Figure 3A-D).

TRB clonality is increased in myelodysplastic syndrome del(5q) bone marrow and GLIPH analysis reveals the presence of myelodysplastic syndrome-specific and lenalidomide-induced T-cell clusters

The BM T-cell space of patients with del(5q) MDS showed a higher *TRB* clonality compared to that of healthy control BM both before and after lenalidomide treatment, while there were no differences in PB T cells suggesting antigen-driven clonal T-cell selection only in the local BM environment (Figure 4A,B).

To further define antigen-driven T-cell dynamics in the BM of del(5q) MDS patients undergoing lenalidomide treatment, we performed GLIPH analysis[27] on our *TRB* NGS dataset. The GLIPH algorithm clusters T-cell receptors according to their presumed antigen specificity based on conserved motif analysis.[26] Since our global analysis of T-cell clonality suggested antigen-driven selection in the BM but not in PB, we exclusively used *TRB* repertoires from the local MDS microenvironment for this analysis. In a first step, T-cell clusters were determined by GLIPH analysis in BM from healthy controls as well as del(5q) MDS patients before and after lenalidomide treatment. When looking at MDS-exclusive T-cell clusters, we found a higher number of newly emerging clusters that were shared between individual patients in the samples after lenalidomide treatment compared to the clusters that were present only at the pre-treatment time point (n=534 *vs.* 240 clusters, respectively) (Figure 5A). This indicated new T- cell cluster generation in response to lenalidomide. Of all clusters that persisted in the BM across the two sampling time points (n=1,242), the majority (n=813) decreased after treatment, while only fewer clusters clonally expanded after treatment (Figure 5A,B). The few MDS-exclusive T-cell clusters that were present before exposure to lenalidomide and that expanded upon treatment were mostly clusters with high generation probability and were therefore less likely to constitute a true T-cell response directed against the MDS clone. In contrast, the T-cell clusters newly generated upon lenalidomide treatment showed much lower generation probabilities, further supporting the hypothesis that the newly emerging clusters (in contrast to the preexisting ones) are specifically generated in response to the MDS clone (Figure 5B). Together, this indicates that despite large overlaps in the pre- and post-therapeutic *TRB* repertoire of individual patients, lenalidomide-induced T-cell immunity may be significantly driven by newly generated T-cell clusters in the local MDS del(5q) BM microenvironment which are shared between individual MDS patients. Table 2 summarizes the presumably most specific top 25 lenalidomide-induced T-cell clusters (low generation probability) that were shared between two or more patients.
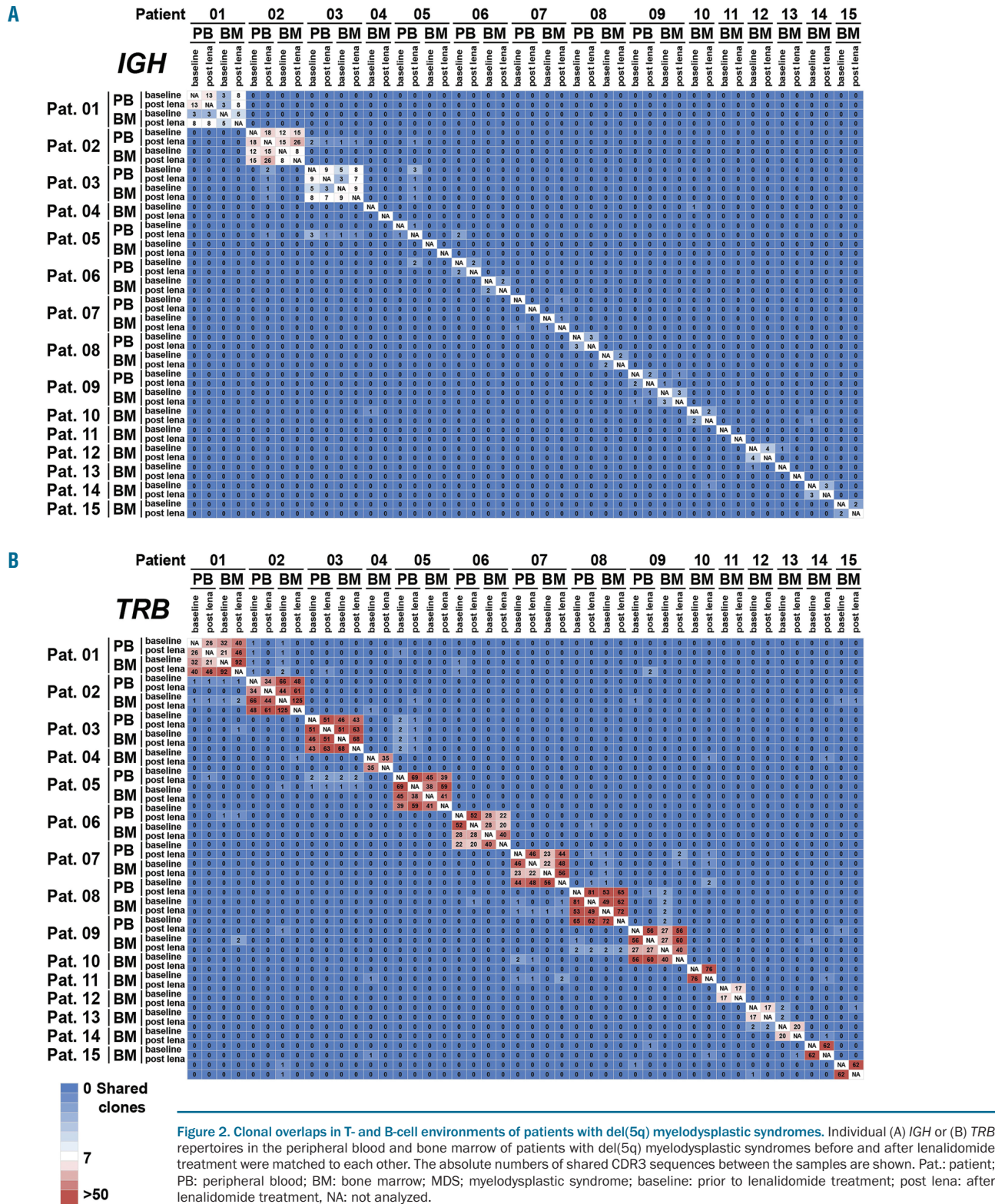
Discussion

The immunomodulatory drug lenalidomide is effective in the treatment of del(5q) MDS. While lenalidomide has undebatable direct effects on the del(5q)-clone mediated by CRBN, the immunomodulatory effects on the microenvironment are less well defined to date. In this study we used state-of-the-art NGS technology to gain insight into local BM immune environments in del(5q) MDS and their modulation upon treatment. NGS technology makes it possible to characterize large immune repertoires in depth and at high throughput, to monitor individual immune clones over time and to integrate all information to derive quantitative and reliable repertoire metrics data such as clonality indices, gene usage, clonal space distribution and somatic mutational frequencies. Thereby, the immune repertoire determined by NGS not only transforms our ability to detect imprints of antigenic drive,[23,30] but also provides valuable insight into immunological drug effects.[24, 31-35]

Our analyses showed that lenalidomide exerts systematic effects on the local B- and T-cell compartments in the BM of patients with del(5q) MDS. Since no significant clonal B-cell overlap between pre- and post-treatment samples was detectable in BM, the small but highly frequent clones detected after initiation of lenalidomide treatment most likely represent newly generated clones, given that our sequencing depth of around 17,000 successfully aligned productive *IGH* reads per sample exceeds the greatest possible number of B lineage cells in the sample (1,136-5,682 per PCR, assuming a percentage of B lineage cells with completed *VDJ* recombination of 3-15% of BM mononuclear cells). Collectively, the changes observed in the *IGH* repertoire reflect the proliferative effect on healthy B-cell precursors previously described for this

drug.[36] It is also in line with the observed upregulation of *PAX5*, a gene involved in *V(H)DJ(H)* recombination of pro-B cells, in lenalidomide responders described in a BM RNA sequencing study of patients with non-del(5q) MDS, suggesting that this drug non-specifically expands the B lineage pool by this mechanism.[19]

The BM T-cell space in patients with del(5q) MDS was significantly more restricted than that of PB indicating T-cell-mediated immunity in the BM niche where T cells are in direct contact with the del(5q) MDS clone. The BM T-cell repertoire was much more conserved over time than the B-cell repertoire with a higher clonal overlap between

Figure 2. Clonal overlaps in T- and B-cell environments of patients with del(5q) myelodysplastic syndromes. Individual (A) *IGH* or (B) *TRB* repertoires in the peripheral blood and bone marrow of patients with del(5q) myelodysplastic syndromes before and after lenalidomide treatment were matched to each other. The absolute numbers of shared CDR3 sequences between the samples are shown. Pat.: patient; PB: peripheral blood; BM: bone marrow; MDS; myelodysplastic syndrome; baseline: prior to lenalidomide treatment; post lena: after lenalidomide treatment, NA: not analyzed.

the pre- and post-therapeutic samples. However, when GLIPH analysis was used to determine the dynamics of T-cell clusters with presumably identical antigen recognition, we found that the majority of preexisting MDS-specific BM T-cell clusters were either lost or reduced in size, while new T-cell clusters with low to medium generation probabilities, which were shared between different patients, emerged upon lenalidomide treatment. Therefore, the cluster analysis provided indirect, but strong evidence that the observed T-cell dynamics is not
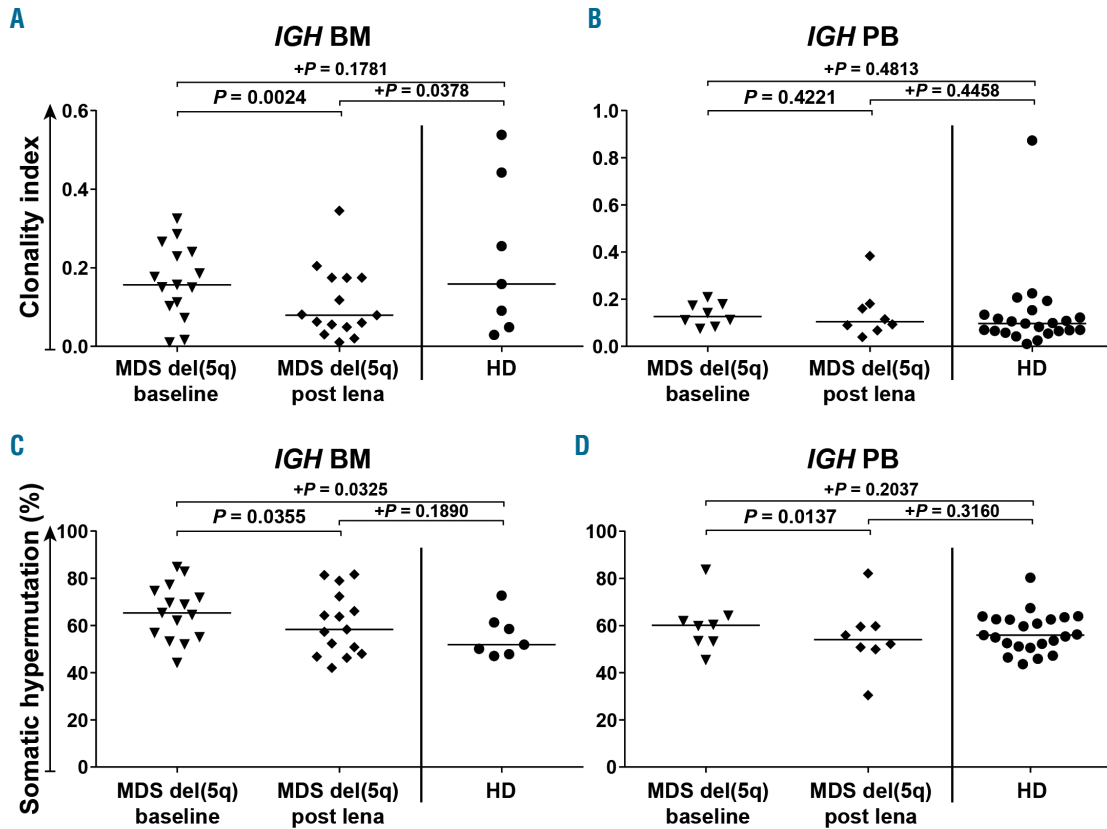

Figure 3. *IGH* clonality and hypermutation in the peripheral blood and bone marrow of patients with myelodysplastic syndromes at baseline and after initiation of lenalidomide treatment. (A,B) Clonality index and (C,D) somatic hypermutation rate of the *IGH* repertoire in samples of (A,C) bone marrow and (B,D) peripheral blood from patients with myelodysplastic syndromes before and after lenalidomide treatment and in samples from healthy controls. The median of each group is indicated in the diagrams. Statistical analysis was performed using the Student *t*-test. Differences between pre- and post-lenalidomide samples are indicated as *P* values and those between either pre- or post-lenalidomide samples and samples from healthy controls are indicated as +*P* values. BM: bone marrow; PB: peripheral blood; MDS; myelodysplastic syndrome; baseline: prior to lenalidomide treatment; post lena: after lenalidomide treatment; HD: healthy donor.
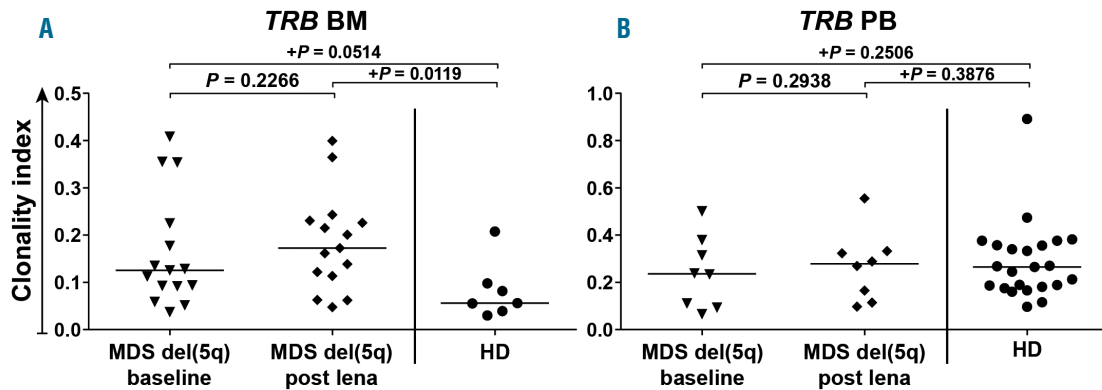

Figure 4. *TRB* clonality in the peripheral blood and bone marrow of patients with del(5q) myelodysplastic syndromes at baseline and after initiation of lenalidomide treatment. (A,B) Clonality index of the *TRB* repertoire in samples of bone marrow (A) and peripheral blood (B) from patients with myelodysplastic syndromes before and after lenalidomide treatment and in samples from healthy controls. The median of each group is indicated in the diagrams. Statistical analysis was performed using the Student *t*-test. Differences between pre- and post-lenalidomide samples are indicated as *P* values and those between either pre- or post-lenalidomide samples and samples from healthy controls are indicated as +*P* values. BM: bone marrow; PB: peripheral blood; MDS; myelodysplastic syndrome; baseline: prior to lenalidomide treatment; post lena: after lenalidomide treatment; HD: healthy donor.

due to non-specific stimulation, but is antigen-driven and directed against the MDS del(5q) clone. Along this line of reasoning, our findings support the concept that the immunomodulatory effect of lenalidomide not only influences the functional state of T cells, but also induces a profound change in the local BM T-cell composition leading to the generation of novel T-cell clusters that can be observed across patients. These clusters, if confirmed by future studies, could also be helpful to explain or even predict resistance or loss of response to lenalidomide.

Moreover, it would be of interest to determine whether these clusters also appear in patients with non-del(5q) disease and represent a general mechanism of action of lenalidomide independently of the del(5q) MDS. This will need to be addressed in future studies.

Expansion of knowledge about the immune response induced by lenalidomide treatment in the BM of MDS patients may be of particular clinical importance in the era of immune checkpoint therapy. Although MDS is considered to be less immunogenic than solid tumors, the obser-
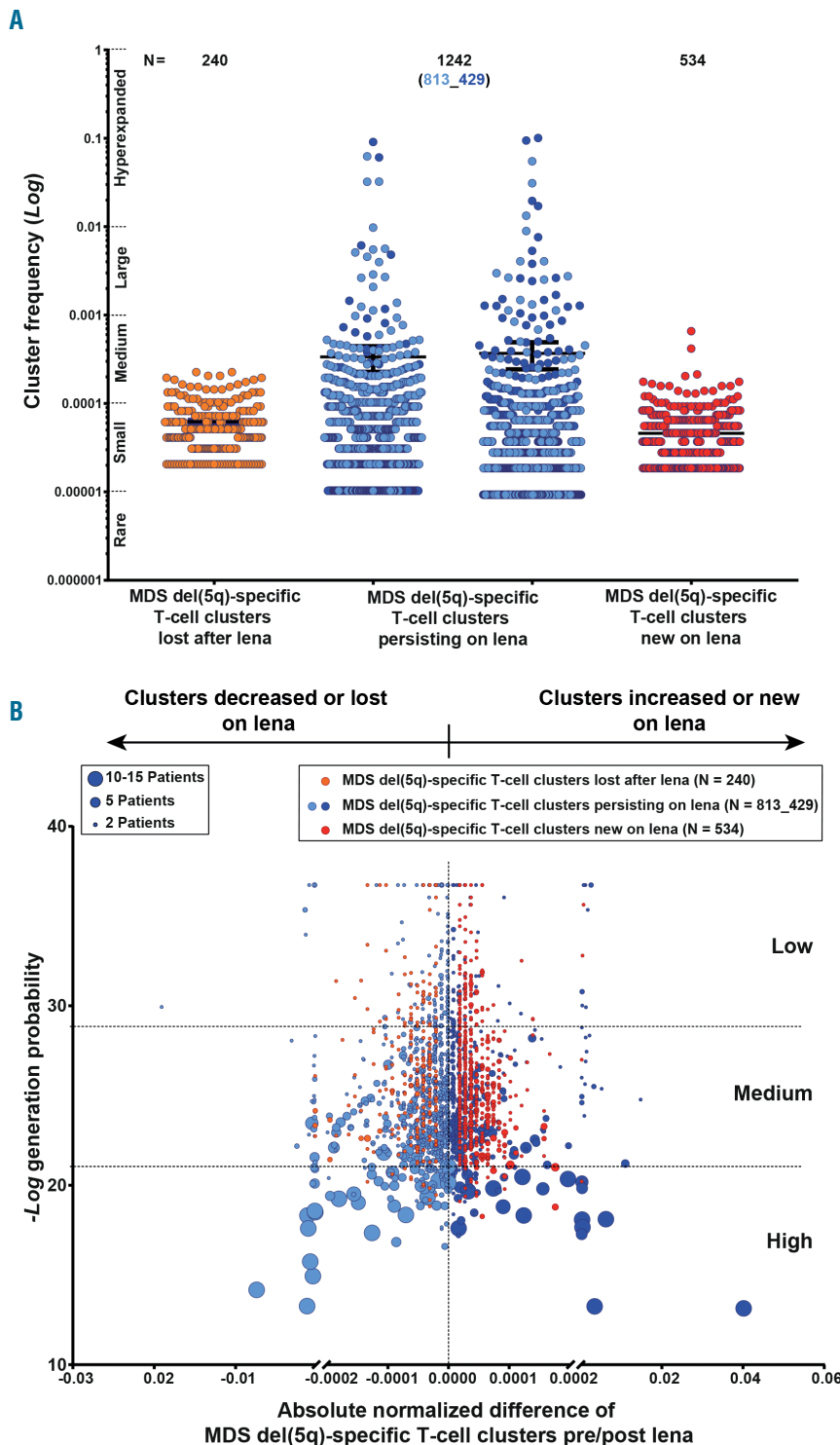

Figure 5. *In silico TRB* cluster analysis of del(5q) myelodysplastic syndrome bone marrow samples at baseline and after initiation of lenalidomide treatment. (A) Frequencies and (B) generation probabilities of *in silico*-generated *TRB* clusters in bone marrow mononuclear cells from patients with del(5q) myelodysplastic syndromes (MDS) at baseline *versus* post-lenalidomide treatment. Clusters detected in both were designated as shared and are colored according to their frequency (bright blue decreasing, dark blue increasing on treatment). The absolute normalized difference of a cluster was calculated by subtracting the cluster frequencies observed in MDS samples at baseline from frequencies observed in samples after lenalidomide treatment. The size of the dots in (B) represents the numbers of individuals sharing the specific cluster. Lena: lenalidomide.

Table 2. Lenalidomide-induced T-cell clusters.

	Cluster (CDR3 sequence)	[a]Post-lenalidomide expansion (absolute normalized difference)	Generation probability
Clusters with low generation probability shared by 3 patients	CASREPSGGKPQHF	5.57E-05	31.88
	CATKDRDSNQPQHF	3.71E-05	31.23
	CASSLWGGGDQPQHF	4.64E-05	30.77
	CASSLGGLAGAEQFF	3.71E-05	30.06
	CATSGSGRFYNEQFF	4.64E-05	29.69
Clusters with low generation probability shared by 2 patients	CASSQDFTDTQYF	1.86E-05	36.74
	CASTEGRADTIYF	1.86E-05	36.74
	CASSLGEGTAGYEQYF	5.57E-05	36.74
	CASSPDGGRGGEQYF	5.57E-05	36.74
	CAWSDRGLIGAFF	1.86E-05	36.74
	CASSSSQAGTEAFF	2.78E-05	36.74
	CASRSRDRGGDTF	1.86E-05	36.74
	CASSLSAGVLSEQYF	3.71E-05	36.74
	CASSSGPRQLFF	3.71E-05	36.04
	CASSLGYPGSASYKQYF	6.59E-04	35.64
	CASSQDSLSGYTF	3.71E-05	35.64
	CSVEALGGATDTQYF	1.86E-05	35.64
	CASSPRGVSGYTF	2.78E-05	34.95
	CASSFPDREQYF	1.86E-05	34.79
	CASSRDRGGVGEKLFF	1.86E-05	34.43
	CASTSGLAVTGELFF	2.78E-05	34.25
	CSASPRPGLAFF	3.71E-05	34.10
	CASRPAGGRGEQFF	4.64E-05	33.60
	CASTTAGAGQPQHF	2.78E-05	33.56
	CASSPSTGGANVLTF	4.64E-05	33.07

[a]The absolute normalized difference is calculated by subtracting the cluster frequencies observed in baseline myelodysplastic syndrome (MDS) samples from those detected in MDS samples after lenalidomide treatment..

vation of novel lenalidomide-related T-cell clusters is reminiscent of recent data on response to PD-1 inhibitors following failure of hypomethylating agent treatment.[37,38] It has been shown that hypomethylating agent therapy leads to upregulation of PD-1 and PD-L1 in MDS BM cells, which may be an explanation of the sensitivity of a subset of MDS patients to immune checkpoint therapy after failure of hypomethylating agent treatment. This concept has not yet been tested in lenalidomide-refractory MDS patients. Our data demonstrating emergence of novel T-cell clusters could, therefore, possibly serve as a novel predictive marker for such a strategy, a possibility which warrants further prospective evaluation. Identification of the antigens recognized by the described lenalidomide-induced shared T-cell clusters could help in the design of novel immunotherapeutic approaches for del(5q) MDS.

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