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Clinical Cancer Research

# Integrated Whole Genome and Transcriptome Analysis Identified a Therapeutic Minor Histocompatibility Antigen in a Splice Variant of *ITGB2*

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## Abstract

**Purpose:** In HLA-matched allogeneic hematopoietic stem cell transplantation (alloSCT), donor T cells recognizing minor histocompatibility antigens (MiHAs) can mediate desired antitumor immunity as well as undesired side effects. MiHAs with hematopoiesis-restricted expression are relevant targets to augment antitumor immunity after alloSCT without side effects. To identify therapeutic MiHAs, we analyzed the *in vivo* immune response in a patient with strong antitumor immunity after alloSCT.

**Experimental Design:** T-cell clones recognizing patient, but not donor, hematopoietic cells were selected for MiHA discovery by whole genome association scanning. RNA-sequence data from the GEUVADIS project were analyzed to investigate alternative transcripts, and expression patterns were determined by microarray analysis and qPCR. T-cell reactivity was measured by cytokine release and cytotoxicity.

**Results:** T-cell clones were isolated for two HLA-B*15:01–restricted MiHA. LB-GLE1-1V is encoded by a nonsynonymous SNP in exon 6 of *GLE1*. For the other MiHAs, an associating SNP in intron 3 of *ITGB2* was found, but no SNP disparity was present in the normal gene transcript between patient and donor. RNA-sequence analysis identified an alternative *ITGB2* transcript containing part of intron 3. qPCR demonstrated that this transcript is restricted to hematopoietic cells and SNP-positive individuals. *In silico* translation revealed LB-ITGB2-1 as HLA-B*15:01–binding peptide, which was validated as hematopoietic MiHA by T-cell experiments.

**Conclusions:** Whole genome and transcriptome analysis identified LB-ITGB2-1 as MiHAs encoded by an alternative transcript. Our data support the therapeutic relevance of LB-ITGB2-1 and illustrate the value of RNA-sequence analysis for discovery of immune targets encoded by alternative transcripts. *Clin Cancer Res; 1–13. ©2016 AACR.*

## Introduction

Patients with hematologic malignancies can be successfully treated with allogeneic hematopoietic stem cell transplantation (alloSCT; ref. 1). Unfortunately, the desired antitumor effect often coincides with undesired side effects against healthy tissues, a complication known as GVHD (2). In HLA-matched alloSCT, donor-derived T cells recognize polymorphic peptides presented by HLA molecules on patient cells. These polymorphic peptides or so-called minor histocompatibility antigens (MiHAs) are not expressed on donor cells due to differences in SNPs between patient and donor. Donor T cells recognizing MiHAs with ubiquitous expression on (non)hematopoietic tissues induce not only the desired antitumor or graft-versus-leukemia (GVL) effect, but also undesired GVHD (3). T-cell depletion of the stem cell graft significantly reduces the incidence and severity of GVHD, but it also diminishes the GVL effect. Therefore, to preserve GVL reactivity, donor T cells are administered after alloSCT as donor lymphocyte infusion (DLI; refs. 2, 4). The development of GVL reactivity after DLI can be accelerated by coadministration of IFNα (5, 6). Analysis of the diversity and tissue distribution of MiHAs targeted in T-cell responses after alloSCT (and DLI) provides insight into the immunobiology of GVL reactivity and GVHD. Moreover, MiHA with restricted expression on cells of the hematopoietic lineage are relevant targets for T-cell therapy to stimulate GVL reactivity after alloSCT without GVHD, as donor T cells for these MiHAs will eliminate all patient hematopoietic cells, including the malignant cells, while sparing healthy hematopoiesis of donor origin.

Since 2009, MiHA discovery accelerated because of development of whole genome association scanning (WGAS). In WGAS as exploited in our laboratory, association between T-cell recognition and SNP genotype is investigated for a panel of 80 EBV-B

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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## Translational Relevance

Hematopoiesis-restricted minor histocompatibility antigens (MiHAs) are relevant targets for immunotherapy, as donor T cells for these antigens stimulate antitumor immunity after HLA-matched allogeneic hematopoietic stem cell transplantation (alloSCT) without undesired side effects. We here identified LB-ITGB2-1 as hematopoiesis-restricted MiHAs that is presented on leukemic cells, but not on nonhematopoietic cells, by a new approach in which whole genome and transcriptome analysis is combined. In this approach, RNA-sequence data were analyzed, and LB-ITGB2-1 was shown to be encoded by an alternative transcript in which intron sequences are retained. Our data show that (i) LB-ITGB2-1 is a relevant antigen to stimulate donor T cells after alloSCT by vaccination or adoptive transfer and (ii) RNA-sequence analysis is a valuable tool to identify immune targets that are encoded by alternative transcripts and created by genetic variants.

cell lines, which have been genotyped for $1.1 \times 10^6$ SNPs (7–14). Associating SNPs as identified by WGAS may directly encode MiHAs or serve as genetic markers for antigen-encoding SNPs that are in linkage disequilibrium with associating SNPs, but which have not been captured by the array. In general, MiHAs can be easily identified by WGAS if one or more associating SNPs are present in coding gene regions. However, antigen discovery is more difficult if associating SNPs are found in genomic regions that are unknown to code for protein. In a number of cases, we sequenced the primary gene transcript as derived from the associating genomic region, but failed to determine any SNP disparity between patient and donor, suggesting that the MiHAs may be encoded by an alternative transcript. MiHAs encoded by alternative transcripts have previously been reported for ACC-6 (15) and ZAPHIR (16). Although MiHA discovery may become more efficient when EBV-B cell lines are used which have been sequenced for their entire genome to increase SNP coverage (17), WGAS identifies a genomic region, and MiHAs encoded by alternative transcripts from these regions may remain difficult to discover.

In this study, we explored whether RNA-sequence data as available in the GEUVADIS project (18, 19) can be used to identify MiHAs encoded by alternative transcripts. With the rapid advances in sequence technology, the GEUVADIS consortium set out to combine whole genome and transcriptome data and sequenced all mRNA expressed in 462 EBV-B cell lines from the 1000 Genomes Project (1000 GP; ref. 20). We analyzed RNA-sequence data to unravel alternative transcripts from *ITGB2* located in a genomic region that has been identified by WGAS for a T-cell clone recognizing an HLA-B*15:01–restricted MiHAs. Our data showed the successful discovery of LB-ITGB2-1 as MiHAs encoded by an alternative *ITGB2* transcript by RNA-sequence analysis. We showed that the alternative *ITGB2* transcript is hematopoiesis-restricted and specifically expressed in SNP-positive individuals. Moreover, T-cell experiments demonstrated specific recognition and lysis of malignant (and healthy) hematopoietic cells, but no reactivity against skin-derived fibroblasts. As such, our data support the therapeutic relevance of LB-ITGB2-1 as hematopoiesis-restricted MiHAs to augment GVL reactivity after alloSCT without GVHD.

## Materials and Methods

### Patient

Patient 6940 (HLA-A*01:01; A*02:01, B*07:02, B*15:01, C*04:01, C*07:02) is a female patient with chronic myeloid leukemia (CML) in blast crisis, who was transplanted with a T-cell–depleted stem cell graft from her HLA-matched brother (donor 7034). She developed a cytogenetic relapse 5.5 months after alloSCT for which she was successfully treated with DLI and IFNα. GVL reactivity after DLI was accompanied with acute GVHD grade II of the skin that evolved into chronic GVHD.

### Cell samples and culture

Peripheral blood, bone marrow, and skin biopsies were obtained from patient 6940, donor 7034, and third-party individuals after approval by the Leiden University Medical Center Institutional Review Board and informed consent according to the Declaration of Helsinki. Peripheral blood and bone marrow mononuclear cells (PBMC and BMMC) were isolated by Ficoll-Isopaque separation and cryopreserved. Fibroblasts (FB), EBV-B cells, and T cells were cultured as described previously (9, 10). In house–generated EBV-B cell lines were authenticated using short-tandem repeat profiling upon freezing of stock vials. FB were cultured in the absence or presence of 200 IU/mL IFNγ (Boehringer-Ingelheim) for 4 days. Patient CML cells in blast crisis in a PBMC sample obtained at diagnosis prior to alloSCT were *in vitro* modified into professional antigen-presenting cells (CML-APC) as described previously (21).

### Isolation of T-cell clones

T cells were isolated from patient PBMC obtained 9 weeks after DLI using the MACS Pan T Cell Isolation Kit according to manufacturer's instructions (Miltenyi Biotec). CML-APC and CD3 T cells were coincubated for 48 hours at a 1:10 stimulator:responder ratio. Cultures were stained with CD8-FITC and CD137-APC mAbs and activated CD137-positive CD8 T cells were single-cell sorted by flow cytometry. Isolated T cells were stimulated with irradiated feeders, phytohemagglutinin, and IL2 as described previously (9). Growing T-cell clones were analyzed for reactivity and restimulated every 14 days as described above.

### T-cell reactivity assays

Stimulator cells ($5–15 \times 10^3$ cells/well) and T cells ($2 \times 10^3$ cells/well) were coincubated overnight in 384-well plates. IFNγ release was measured by ELISA (Sanquin). In chromium-release assays, target cells ($1 \times 10^3$ cells/well) were labeled for 1 hour at 37°C with 100 μCi (3.7 MBq) $Na_2{}^{51}CrO_4$ and coincubated with T cells for 10 hours at a 10:1 effector:target (E:T) ratio. Specific lysis was calculated as described previously (10).

### Whole genome association scanning

SNP-genotyped EBV-B cell lines ($n = 71$) were transduced with an LZRS retroviral vector (22) encoding HLA-B*15:01. Mean transduction efficiency was 24% (range, 12%–34%), and T-cell recognition was measured by IFNγ ELISA. WGAS was performed as described previously (9).



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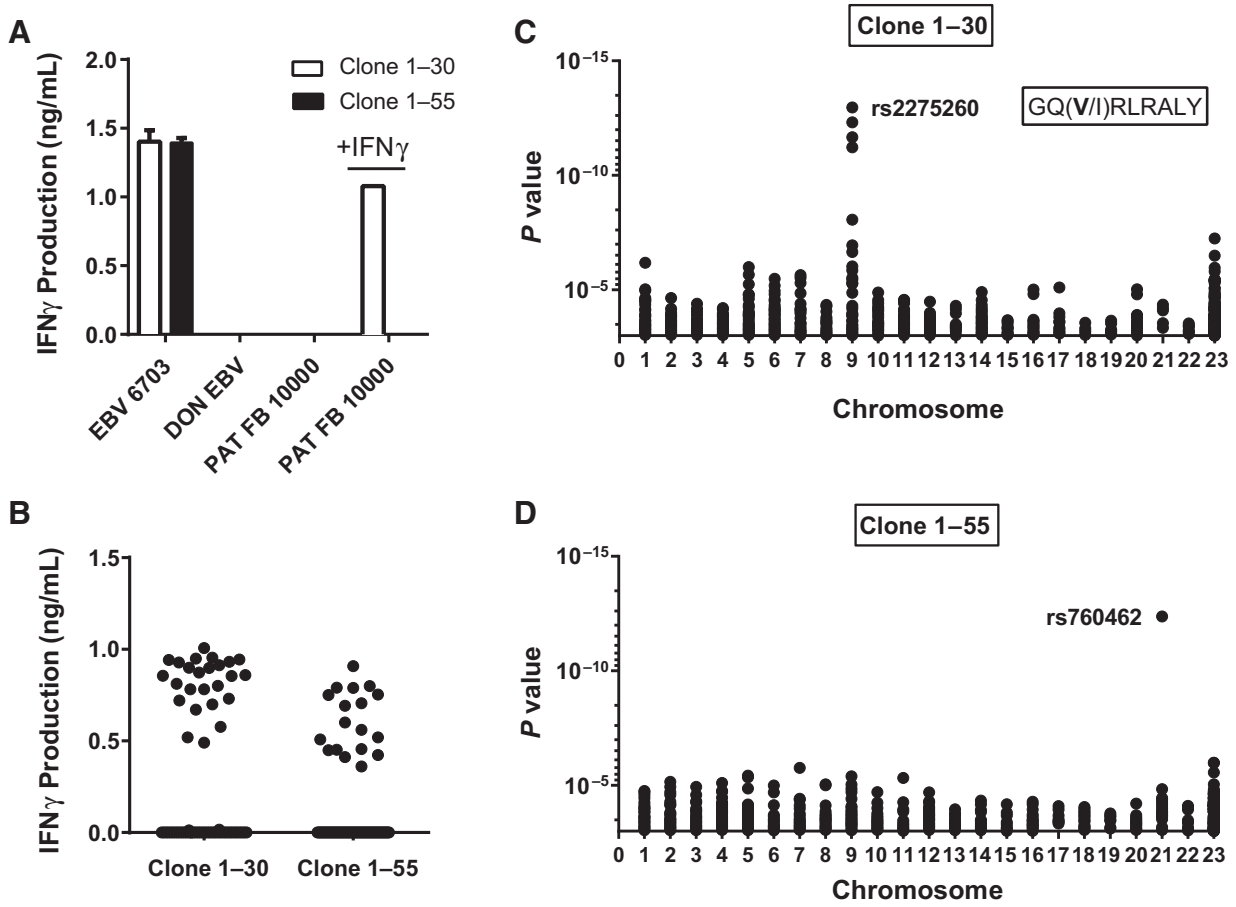


**Figure 1.**

T-cell clones 1–30 and 1–55 recognize HLA-B*15:01–restricted minor histocompatibility antigens. A, reactivity of T-cell clones 1–30 (open bars) and 1–55 (filled bars) was measured against patient FB, which were cultured in the absence or presence of IFNγ, and against HLA-B*15:01-positive, MiHA-positive, and MHA-negative EBV-B cells (third party EBV 6703 and DON EBV, respectively). T-cell reactivity was measured by IFNγ ELISA after overnight coincubation. B, recognition of a panel of 71 SNP-genotyped EBV-B cell lines after retroviral transduction with HLA-B*15:01 was measured for T-cell clones 1–30 and 1–55. Each symbol represents IFNγ production as measured by ELISA after overnight coincubation of the T-cell clone with each individual EBV-B cell line. C and D, association was measured between T-cell recognition of 71 EBV-B cell lines transduced with HLA-B*15:01 and each of the $1.1 \times 10^6$ SNPs as measured by the Illumina 1M array. SNPs are grouped by their location on chromosomes (*x*-axis). SNPs with *P* value $<10^{-3}$ are displayed. C, whole genome association scanning results for clone 1–30 are depicted. Strong association was found for SNP rs2275260 ($P = 1.08 \times 10^{-15}$) located in exon 6 of the *GLE1* gene, which encodes an amino acid change in a peptide with strong predicted binding to HLA-B*15:01 by NetMHC: GQ(V/I)RLRALY. D, whole genome association scanning results for clone 1–55 are depicted. Strong association was found for SNP rs760462 ($P = 4.26 \times 10^{-13}$) located in intron 3 of the *ITGB2* gene.

### RNA-sequence analysis

EBV-B cell lines for which RNA-sequence data (.bam files) are available in the GEUVADIS project (18, 19) were selected for SNP genotype (+/+, +/−, and −/−) from the 1000 GP (20). For each SNP (rs760462 and rs9945924), two representative individuals per genotype were selected, and bigwig files containing RNA-sequence coverage and mapping and split coordinates of individual sequence reads in the region of interest for these EBV-B cell lines were uploaded to the UCSC genome browser (23).

### Prediction of HLA-binding peptides

Transcript sequences were translated in forward-reading frames, and protein sequences were fed into the NetMHC algorithm (24, 25) to search for peptides with predicted binding to HLA class I alleles. Peptides were synthesized, dissolved in DMSO, and tested for T-cell recognition by IFNγ ELISA (9).

### Microarray gene expression and qPCR analysis

Malignant and healthy hematopoietic cells and untreated as well as IFNγ pretreated nonhematopoietic cells of different origins were processed and hybridized on Human HT-12 v3/4 Expression BeadChips (Illumina) as described previously (26). The data have been deposited in NCBI's Gene Expression Omnibus (27) and are accessible through GEO Series accession number GSE76340 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76340). For quantitative RT-PCR, RNA isolation, cDNA synthesis and qPCR were performed as described previously (28). *ITGB2* expression was measured using forward primer 5′-CTCTCTCAGGAGTGCACGAA-3′ and reverse primer 5′-CCCTGTGAAGTTCAGCTTCTG-3′ for the normal *ITGB2* transcript and forward primer 5′-CAGCAGCTG-CCGGGAATG-3′ and reverse primer 5′-CTCAGTCCGAGGACA-GACGG-3′ for the alternative *ITGB2* transcript. Expression was normalized for expression of the *HMBS* reference gene.



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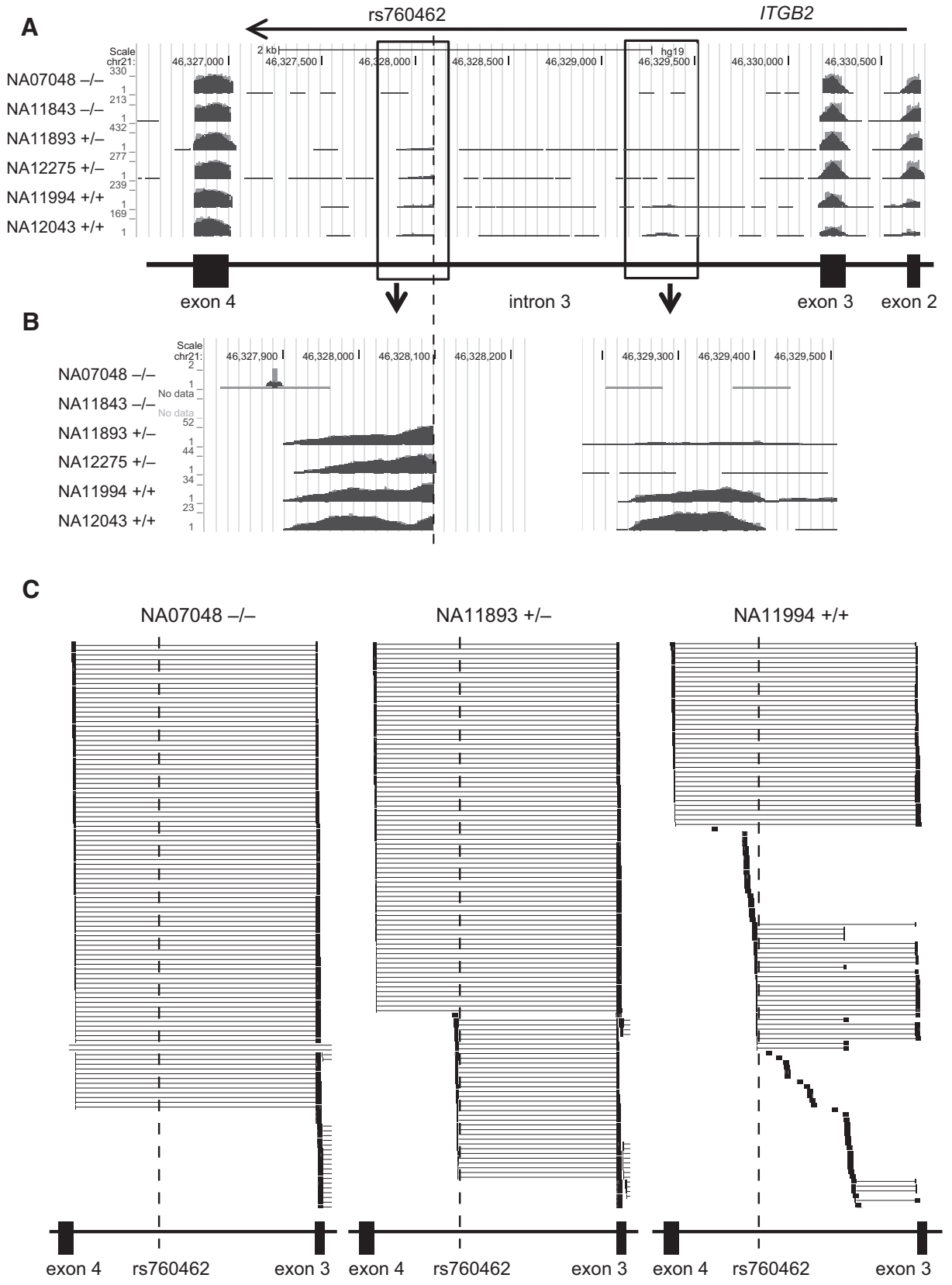




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### Colony-forming assay

Bone marrow or peripheral blood samples from patients with CML were preincubated with irradiated (35 Gy) T-cell clones at E:T ratios of 3:1. After overnight coincubation, single-cell suspensions ($2 \times 10^4$ target cells) were seeded in 30-mm culture dishes containing IMDM with methylcellulose and growth factors (GM-CSF, stem cell factor, IL3, erythropoietin, and other supplements (MethoCult H4434, STEMCELL Technologies SARL). As controls, single-cell suspensions and irradiated T cells at E:T ratios of 3:1 were seeded without preincubation. After 14 days of culture, colony-forming units for granulocyte/macrophage (CFU-GM, CFU-G, CFU-M) and erythroid (CFU-E, BFU-E) lineages were enumerated as well as colony-forming units containing early progenitors that differentiated to granulocyte/macrophage/erythroid lineages.

## Results

### Isolation of T-cell clones for HLA-B*15:01–restricted MiHAs

To identify MiHAs that are targeted in effective GVL responses, CD8 T-cell clones were isolated from a patient with CML in blast crisis who entered into complete remission upon treatment with DLI after HLA-matched alloSCT. Development of antitumor immunity after DLI was accompanied with grade II skin GVHD. CD3 T cells isolated from patient PBMC after DLI were stimulated with a patient CML sample obtained at diagnosis prior to alloSCT. This sample consisted of a heterogeneous population, containing 63% mature myelocytes, 17% immature CD34-negative cells, and 17% immature CD34-positive cells. To enhance antigen presentation by the stimulator cells, patient CML cells were modified *in vitro* into professional APC (CML-APC). After 48 hours of stimulation, CD8 T cells were single cell sorted by flow cytometry based on the expression of activation marker CD137. Growing CD8 T-cell clones ($n = 112$) were tested for reactivity against patient CML-APC, donor EBV-B, donor EBV-B pulsed with a mix of known MiHA peptides and patient FB, which were cultured in the absence or presence of IFNγ to mimic the inflammatory environment of the early posttransplantation period. T-cell clones recognizing known MiHA peptides were specific for LB-ADIR-1F (29) and LRH-1 (30). T-cell clones 1–30 and 1–55 recognized unknown MiHAs.

T-cell clones 1–30 and 1–55 both showed strong reactivity against patient CML-APC (data not shown) as well as EBV-B cells from an HLA-B*15:01 third-party individual, but not against donor EBV-B (Fig. 1A). In contrast to clone 1–30 that strongly recognized patient FB after pretreatment with IFNγ, T-cell clone 1–55 lacked reactivity against (IFNγ pretreated) FB. To identify the epitopes that are recognized by clones 1–30 and 1–55, WGAS was performed to investigate association between T-cell recognition and all individual SNPs as measured by the array (Fig. 1B; ref. 9). For clone 1–30, WGAS identified four SNPs that associated with T-cell recognition of HLA-B*15:01-transduced EBV-B cells with the same *P* value of $1.08 \times 10^{-13}$ (Fig. 1C). These four SNPs were located in a genomic region on chromosome 9 comprising the *GLE1* gene. One nonsynonymous SNP (rs2275260) in exon 6 encoded an amino acid change in the GLE1 protein. Patient and donor peptides GQ(V/I)RLRALY with predicted binding to HLA-B*15:01 by NetMHC (24, 25) were identified and the V-variant (LB-GLE1-1V) was recognized by clone 1–30.

For clone 1–55, WGAS identified a single SNP on chromosome 21 with a *P* value of $4.26 \times 10^{-13}$ (Fig. 1D). This SNP rs760462 A/G (A; recognized allele) is located in intron 3 (region between exon 3 and exon 4) of the *ITGB2* gene. Sanger sequencing of the normal *ITGB2* transcript did not detect any SNP differences between patient and donor. In addition, intron sequences comprising rs760462 were translated *in silico* in different reading frames, but no peptide with predicted binding to HLA-B*15:01 was found (data not shown).

### Whole transcriptome analysis enabled discovery of a MiHA encoded by an alternative *ITGB2* gene transcript

As no SNP differences were found between patient and donor in the normal *ITGB2* transcript, we explored the possibility that the MiHAs as recognized by clone 1–55 may be encoded by an alternative transcript. RNA-sequence data were analyzed as online available in the GEUVADIS project (18, 19) for 462 EBV-B cell lines for which corresponding whole genome sequences are available in the 1000 GP. On the basis of SNP genotypes, EBV-B cell lines were selected from individuals who were homozygous positive (A/A; +/+), heterozygous (A/G; +/−), or homozygous negative (G/G; −/−) for associating SNP rs760462. For 6 individuals, RNA-sequence reads were aligned with the *ITGB2* gene in the human HG19 reference genome. Figure 2A depicts transcriptional activity summarized as RNA-sequence read coverage surrounding SNP rs760462. Significant numbers of reads

**Figure 2.**

Discovery of an alternative *ITGB2* gene transcript by whole transcriptome analysis. *ITGB2* is located on chromosome 21q22.3 and is encoded on the reverse strand. Graphs (A–C) are screenshots from the UCSC genome browser at http://genome.ucsc.edu. Exons are indicated by black rectangles. The genomic location of associating SNP rs760462 as identified by WGAS is indicated by the vertical dotted lines. A, RNA-sequence reads aligning with exon 2 to 4 of the *ITGB2* gene in the human HG19 reference genome are shown for 6 individuals representing different genotype groups for associating SNP rs760462 (+/+, +/−, and −/−). The *y*-axis indicates the total number of RNA-sequence reads aligning with the indicated *ITGB2* gene region as summarized peaks. Substantial numbers of RNA-sequence reads aligned with exon regions of the *ITGB2* gene in all 6 individuals irrespective of genotyping for SNP rs760462. B, enlarged view with the *y*-axis adjusted to a lower range of RNA-sequence reads is shown for two regions in intron 3 that are transcriptionally active, as indicated by black rectangles in A. The intron 3 region located upstream (right) of associating SNP rs760462 was transcribed in individuals who were +/+ for this SNP, but not in individuals who were −/− for this SNP. As this region is not transcribed in +/− individuals, we concluded that this region is unlikely to encode the MiHA. In contrast, the intron 3 region located downstream of associating SNP rs760462 (left) was transcribed in both +/− and +/+ individuals, but not in −/− individuals, and we therefore analyzed this region in more detail. C, single RNA-sequence reads aligning within the gene region spanning exon 3 to exon 4 of *ITGB2* in the human HG19 reference genome are shown for 3 individuals representing different SNP genotypes (+/+, +/−, and −/−). RNA-sequence reads that aligned with continuous 75 bp sequences that do not cross exon boundaries (exon reads) were excluded from the analysis. All other RNA-sequence reads, which included intron reads and split reads, are shown. Split reads are indicated by two boxes connected with a horizontal line and are the result of distinct genomic sequences that are joined in a transcript due to splicing. All split reads in −/− individuals contained exon 3 connected to exon 4, indicating expression of the normal *ITGB2* gene transcript. In addition to split reads for the normal gene transcript, split reads in which exon 3 was connected to intron 3 sequences located downstream of the associating SNP were measured in +/− and +/+ individuals. These data indicate that in addition to the normal gene transcript, an alternative *ITGB2* transcript in which part of intron 3 is retained is expressed in individuals who are positive for associating SNP rs760462.



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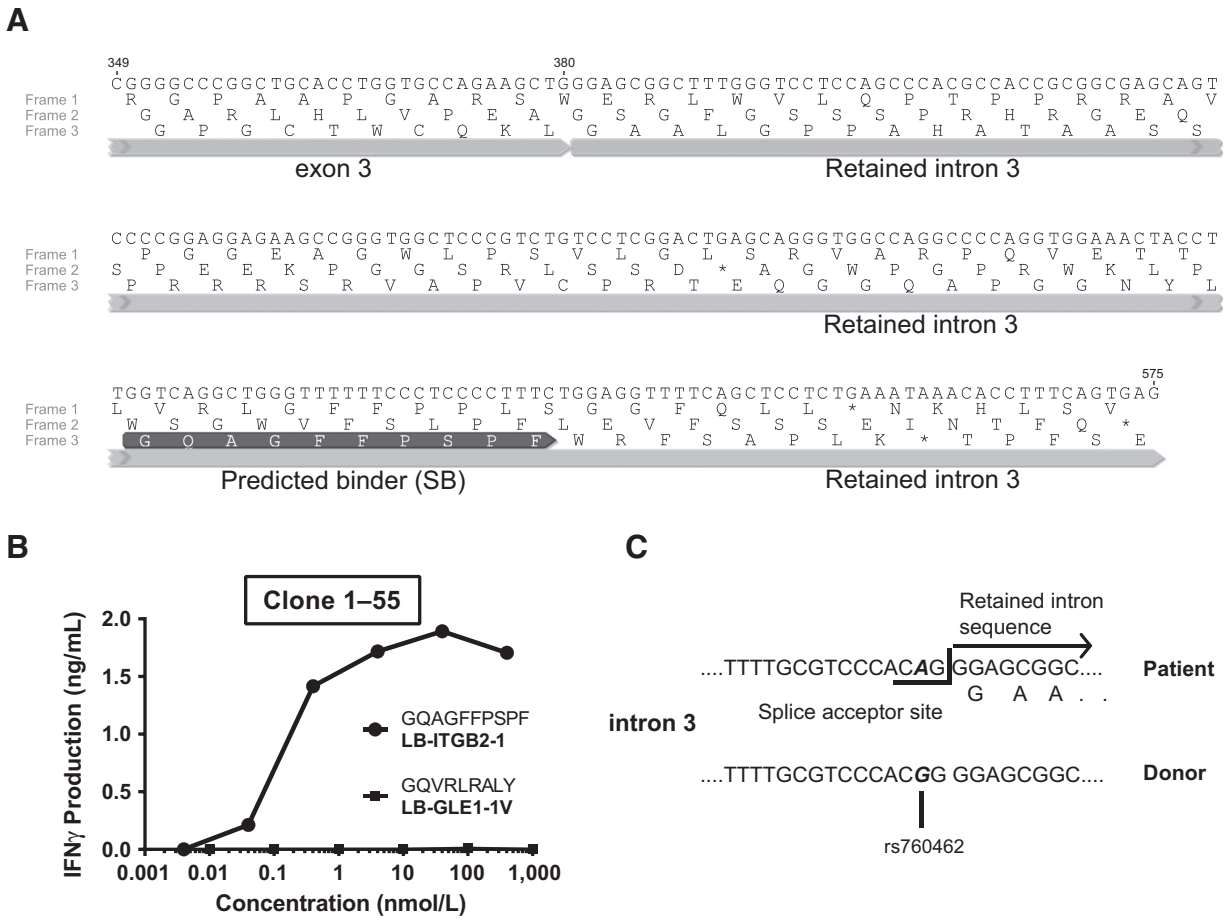


**Figure 3.**
Discovery of a MiHA that is encoded by the alternative *ITGB2* gene transcript. A, the exact sequence composition of the alternative *ITGB2* transcript in which exon 3 is connected to a region in intron 3 located downstream from associating SNP rs760462 was deduced from split read analysis as shown in Fig. 2C. The alternative transcript was translated and a peptide with strong predicted binding (SB) to HLA-B*15:01 as revealed by NetMHC is depicted by the dark gray bar. Sequences are depicted using Geneious (version 7.1.5 created by Biomatters, available from http://www.geneious.com/). B, identification of the HLA-B*15:01-restricted epitope as recognized by T-cell clone 1–55. Peptide GQAGFFPSPF, which has been identified as peptide with strong predicted binding to HLA-B*15:01 by NetMHC, was titrated on donor EBV-B cells and coincubated with T-cell clone 1–55. T-cell recognition after overnight coincubation was measured by IFNγ ELISA. C, intron 3 sequences of *ITGB2* for patient and donor including rs760462 (in bold) are depicted. SNP rs760462 likely creates a splice acceptor site (C*A*G), resulting in retention of intron sequences located 2 nucleotides downstream from the SNP in patient, but not donor.

aligned with exon regions in the *ITGB2* gene, but also two regions in intron 3 were transcribed. The intron region located upstream of associating SNP rs760462 (Fig. 2B, right) was transcriptionally active in individuals who were +/+, but not in individuals who were +/− or −/− for this SNP. As this region was not transcribed in +/− individuals, we considered it unlikely that this region encoded the MiHA. In contrast, the intron 3 region downstream from rs760462 was transcribed in both +/− and +/+, but not in −/− individuals (Fig. 2B, left) and we therefore investigated this region in more detail.

To determine the sequence composition of the alternative transcript, we evaluated alignments of split reads in the region that spanned exon 3 to exon 4 (Fig. 2C). Split reads are the result of distinct genomic sequences that are joined in a transcript due to splicing. In −/− individuals, all split reads contained exon 3 connected to exon 4, indicating expression of the normal *ITGB2* transcript. In contrast, in +/− and +/+ individuals, split reads for the normal *ITGB2* transcript were found as well as split reads in which exon 3 was connected to intron 3 sequences located two nucleotides downstream from the associating SNP. These data

**Table 1.** Predicted peptides for ITGB2

| Frame | Length (aa) | Peptide | Log score | Affinity (nmol/L) | Bind level |
|---|---|---|---|---|---|
| 2 | 9 | GQAPGGNYL | 0.545 | 136 | WB |
| 2 | 10 | GQAGFFPSPF | 0.735 | 17 | SB |
| 2 | 11 | EQGGQAPGGNY | 0.435 | 451 | WB |
| 2 | 11 | LGQAGFFPSPF | 0.613 | 65 | WB |



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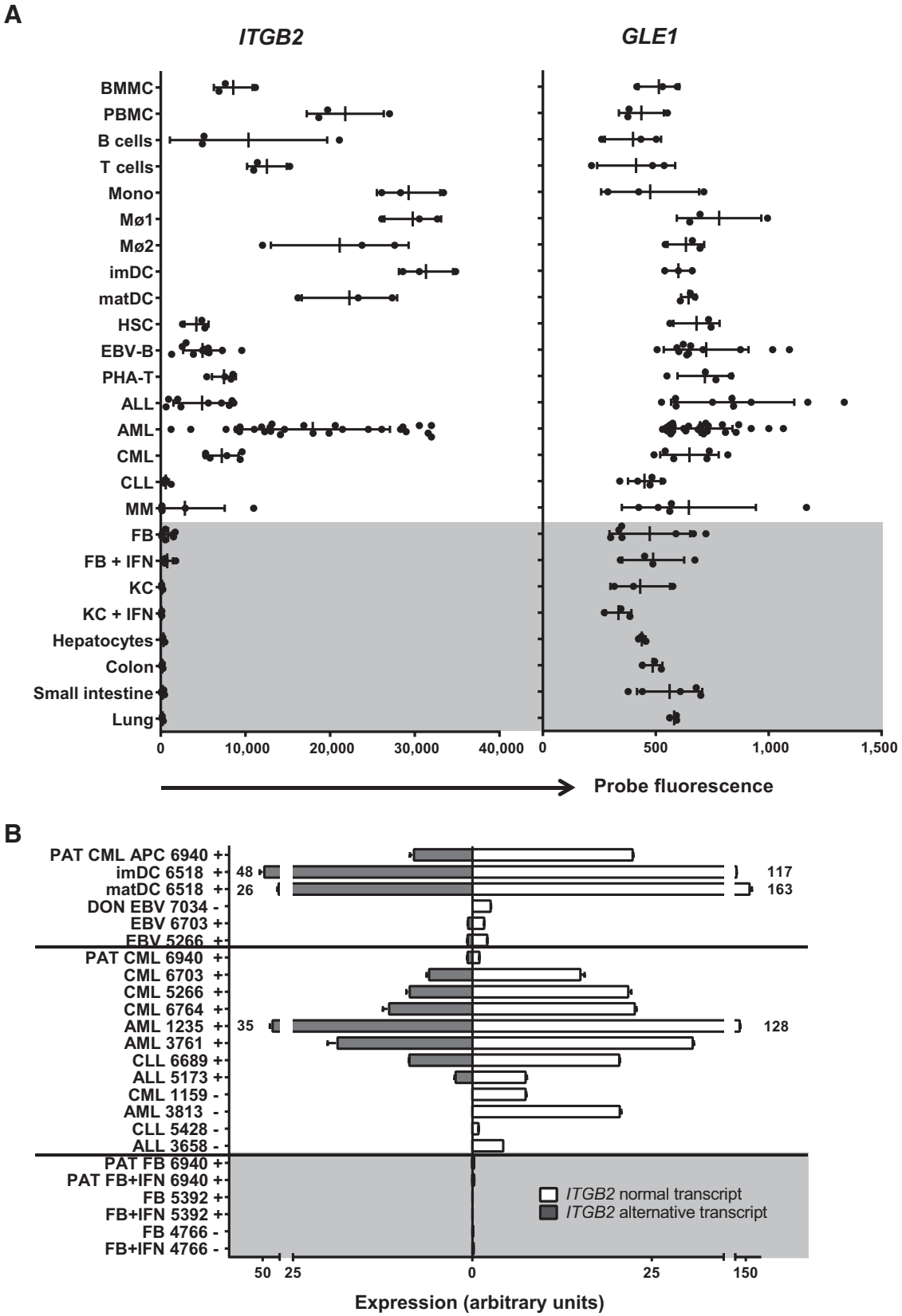




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demonstrate expression of an alternative *ITGB2* gene transcript in which part of intron 3 is retained. Expression of the alternative transcript was restricted to SNP-positive individuals, likely due to rs760462 introducing a cryptic splice acceptor site.

Next, we translated the alternative *ITGB2* transcript *in silico* (Fig. 3A) and protein sequences starting from exon 3 were fed into the NetMHC algorithm (24, 25) to identify peptides with predicted binding to HLA-B*15:01. Four peptides were identified (Table 1) including one 10-mer peptide with strong predicted binding to HLA-B*15:01. T-cell experiments confirmed that GQAGFFPSPF is the MiHA (LB-ITGB2-1) that is recognized by clone 1–55 with picomolar affinity (Fig. 3B). In conclusion, RNA-sequence analysis revealed an alternative *ITGB2* transcript in which associating SNP rs760462 generates a splice acceptor site, thereby creating a transcript in which part of intron 3 is retained (Fig. 3C). This alternative transcript encoded the MiHA recognized by T-cell clone 1–55. As such, whole transcriptome analysis enabled discovery of LB-ITGB2-1 as MiHA encoded by an alternative *ITGB2* transcript.

### Whole transcriptome analysis also allows discovery of antigens generated by exon skipping

To explore the value of RNA-sequence analysis for identification of antigens beyond LB-ITGB2-1, we analyzed RNA-sequence data for ACC-6, an HLA-B*40:01-restricted MiHA encoded by an *HMSD* splice variant (15). Expression of ACC-6 is associated with SNP rs9945924 in intron 2 of *HMSD* located 5 bp downstream of exon 2. We selected EBV-B cell lines from GEUVADIS and compared *HMSD* gene transcription between individuals with different genotypes for the associating SNP (+/+, +/−, and −/−). In contrast to *ITGB2*, no transcriptional activity was found outside exon regions of *HMSD* (Supplementary Fig. S1A). Furthermore, we noticed that exon 2 was not transcribed in +/+ individuals, whereas transcription was clearly detectable in −/− and +/− individuals, indicating that SNP rs9945924 is associated with exon 2 skipping. Split read analysis revealed that only the full-length *HMSD* transcript was expressed in −/− individuals, whereas both the full-length transcript as well as an alternative transcript in which exon 1 was connected to exon 3 were detectable in +/− individuals (Supplementary Fig. S1B). In +/+ individuals, only the alternative transcript was expressed in which exon 2 is skipped. *In silico* translation of the full-length and alternative *HMSD* transcripts revealed that exon 2 skipping deleted the ATG start site, thereby producing a shorter protein in an alternative reading frame (Supplementary Fig. S2). We searched the alternative protein for peptides with predicted binding to HLA-B*40:01 and identified 8 peptides (Supplementary Table S1), including ACC-6 epitope MEIFIEVFSHF (15), in which M is encoded by the first start codon in the alternative transcript. These data demonstrate that RNA-sequence analysis also allows discovery of antigens encoded by alternative transcripts that are generated by exon skipping.

### T cells for LB-ITGB2-1 were detected after DLI

Next, we investigated the *in vivo* immunodominance of LB-ITGB2-1 during GVL reactivity and compared the T-cell frequency for LB-ITGB2-1 at 9 weeks after DLI with other MiHAs that were targeted in patient 6940 (LB-ADIR-1F, LRH-1, and LB-GLE1-1V). Tetramer analysis in Supplementary Fig. S3 shows that T cells for LB-ADIR-1F (0.46%), LRH-1 (0.35%), LB-ITGB2-1 (0.14%), and LB-GLE1-1V (0.08%) are involved in the immune response after DLI. T-cell frequencies prior to DLI were absent or significantly lower for all MiHAs, indicating induction and expansion of a polyclonal T-cell response targeting multiple MiHAs during GVL reactivity.

### Expression of the alternative *ITGB2* transcript is hematopoiesis-restricted

As T-cell clone 1–55 recognized patient CML-APC, but failed to react with FB (Fig. 1A), and expression of *ITGB2* has been reported to be hematopoiesis-restricted (31), we investigated whether T cells for LB-ITGB2-1 could contribute to GVL responses. Microarray gene expression analysis (26) confirmed that the normal *ITGB2* transcript was expressed in malignant and healthy hematopoietic cells, but not in nonhematopoietic cells from organs that are often targeted by GVHD (skin, liver, gut, lung; Fig. 4A). In contrast, the *GLE1* gene, which encodes the MiHA recognized by clone 1–30, was ubiquitously expressed in (non)hematopoietic tissues.

As LB-ITGB2-1 is encoded by a splice variant, we investigated the tissue distribution of this alternative transcript and compared expression with the normal transcript by quantitative RT-PCR. In line with RNA-sequence data (Fig. 2), the alternative transcript was only detected in SNP rs764062-positive individuals, whereas the normal transcript was measured in all individuals irrespective of SNP genotype (Fig. 4B). In all hematopoietic samples from SNP-positive individuals, expression of the *ITGB2* splice variant followed the same pattern as the normal transcript. Moreover, both normal and alternative transcripts were undetectable in nonhematopoietic (IFNγ pretreated) FB, indicating that these gene products are regulated by the same hematopoiesis-restricted transcriptional control elements.

### T cells for LB-ITGB2-1 showed specific recognition and lysis of primary leukemic cells

To investigate whether LB-ITGB2-1 has therapeutic potential, we selected leukemic cells of different origins and compared T-cell

**Figure 4.**

Expression of the alternative *ITGB2* transcript is hematopoiesis-restricted. A, expression of *ITGB2* and *GLE1* as determined by microarray gene expression analysis. *GLE1* is broadly expressed, whereas expression of *ITGB2* is hematopoiesis-restricted. Indicated is the probe fluorescence as measured on Illumina Human HT-12 v3/4 BeadChips. Hematopoietic cells included bone marrow and peripheral blood mononuclear cells (BMMC and PBMC), B cells, T cells, monocytes (Mono), macrophages type I and II (MØ1 and MØ2), (im)mature DC (imDC and matDC), hematopoietic stem cells (CD34 HSC), EBV-B, and PHA-T cells. Malignant hematopoietic cells included acute lymphoblastic leukemia (CD19 ALL), acute myeloid leukemia (CD33 AML), chronic myeloid leukemia (CD34 CML), chronic lymphocytic leukemia (CD5 CD19 CLL), and multiple myeloma (CD138 MM), which were sorted by flow cytometry based on the indicated markers. Nonhematopoietic cells as indicated by the shaded panel included skin FB and KC which were cultured in the absence or presence of IFNγ, hepatocytes, colon, and small intestine epithelial cells and lung epithelial cells. B, expression of normal and alternative *ITGB2* transcripts as measured by quantitative RT-PCR in various cell types. Malignant hematopoietic samples included unselected PBMC or BMMC samples obtained at diagnosis with >30% malignant cells. Genotyping results (+ or −) for LB-ITGB2-1 associating SNP rs760462 are depicted. Expression of the normal (open bars) and alternative (filled bars) *ITGB2* transcripts is shown after correction for expression of the *HMBS* reference gene in arbitrary units.



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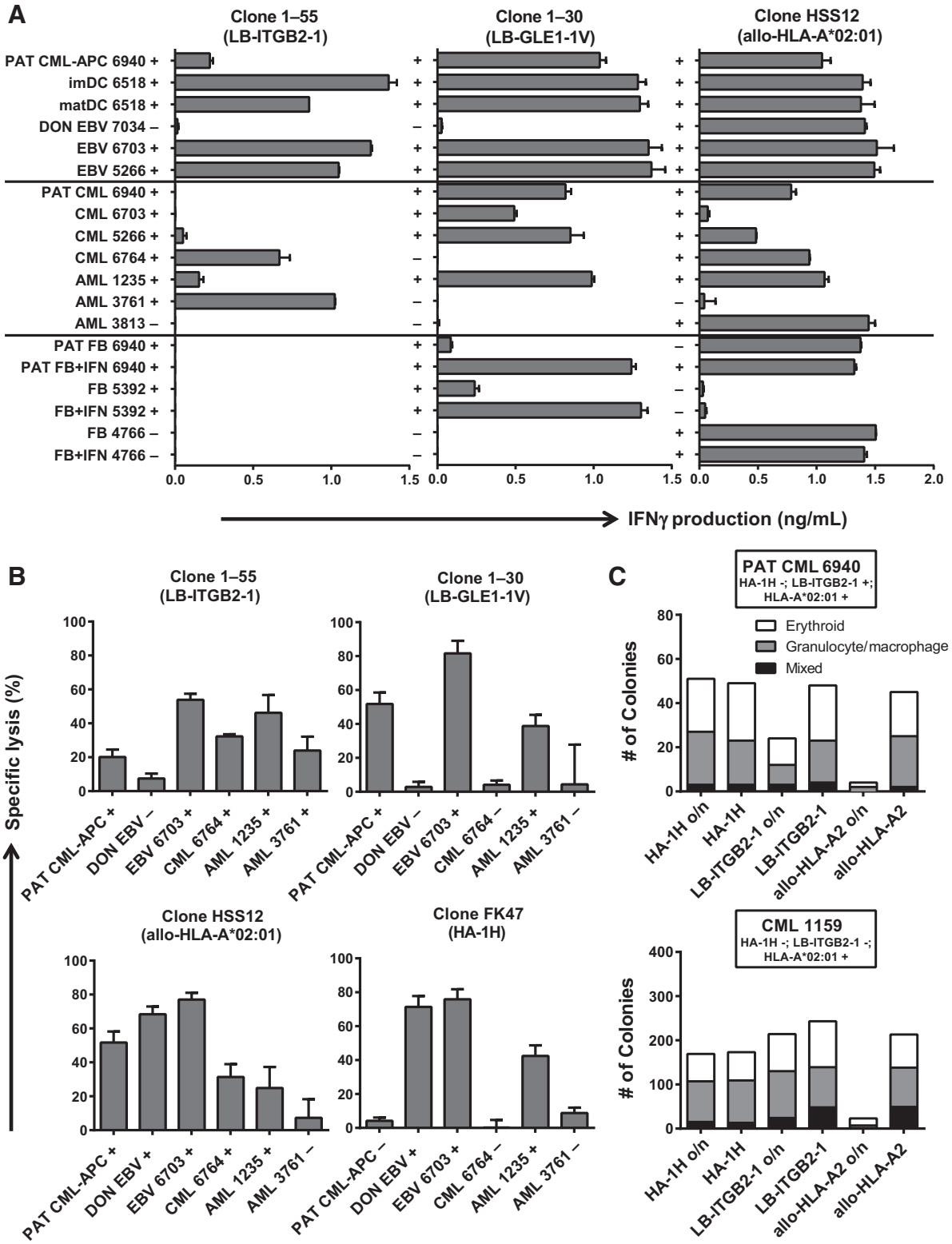


**Figure 5.**

T cells for LB-ITGB2-1 showed specific recognition and lysis of primary leukemic cells. A, reactivity of T-cell clones 1–55 (left) and 1–30 (middle) was measured against CML-APC and FB cultured in the absence or presence of IFNγ of patient 6940 as well as patient PBMC obtained at diagnosis (PAT CML) and samples from other HLA-B*15:01 patients suffering from CML or AML who were positive for SNP rs760462 (LB-ITGB2-1) or rs2275260 (LB-GLE1-1V). Donor EBV-B cells and third party HLA-B*15:01 individuals negative for one or both SNPs were included as controls. (*Continued on the following page.*)



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recognition of these samples as measured by IFNγ ELISA with FB cultured in the absence or presence of IFNγ. Figure 5A shows that clone 1–55 recognized patient CML-APC as well as EBV-B cells and (im)mature DC from HLA-B*15:01 individuals who were positive for SNP rs760462 (left). EBV-B cells were strongly recognized by clone 1–55, whereas *ITGB2* gene expression was low (Fig. 4B), which can be explained by high surface expression of HLA class I, costimulatory and adhesion molecules as well as other molecules involved in intracellular antigen processing and presentation. In addition, various CML and AML samples from SNP-positive patients were recognized, whereas the T-cell clone failed to recognize (IFNγ pretreated) patient FB as well as FB from another SNP-positive individual. Clone 1–55 also failed to recognize patient CML cells obtained at diagnosis and CML cells from another SNP-positive patient. Both CML samples expressed low levels of the alternative *ITGB2* transcript by quantitative RT-PCR (Fig. 4B). One AML sample (AML 1235) was only moderately recognized by clone 1–55, whereas the *ITGB2* gene was highly expressed, which can be due to suboptimal expression of HLA class I or other accessory molecules in antigen processing and presentation.

Furthermore, we showed that T cells for LB-ITGB2-1–mediated specific lysis of primary leukemic cells in a 10-hour chromium-release assay (Fig. 5B, top left). T-cell clone 1–55 mediated specific lysis of patient CML-APC, whereas donor EBV-B cells were not lysed. In addition, EBV-B cells as well as AML and CML samples from other SNP-positive individuals were lysed.

To investigate the capacity of LB-ITGB2-1–specific T cells to recognize primary AML cells directly *ex vivo*, we performed a flow cytometry experiment in which we measured upregulation of activation marker CD137 on LB-ITGB2-1 tetramer-positive T cells as circulating in peripheral blood after DLI. The data showed significant upregulation of CD137 on T-cell clone 1–55 after 36 hours of stimulation with unmanipulated AML cells that were positive for LB-ITGB2-1 and HLA-B*15:01 as compared with HLA-B*15:01 positive AML cells that were negative for LB-ITGB2-1 (Supplementary Fig. S4). However, numbers of LB-ITGB2-1 tetramer-positive T cells in peripheral blood were too low to draw firm conclusions.

Finally, we performed a colony-forming assay to investigate the capacity of LB-ITGB2-1–specific T cells to kill the malignant hematopoietic progenitor cells as present in peripheral blood from our patient with CML at diagnosis. After overnight coincubation of CML precursor cells with clone 1–55, a 50% reduction was measured in number of colonies differentiated into the granulocyte/macrophage (CFU-GM, CFU-G, and CFU-M) or erythroid (CFU-E and BFU-E) lineage as well as in number of colonies from more early progenitor cells containing a mixture of cells differentiated into granulocyte/macrophage/erythroid lineages (Fig. 5C). No decrease in number of colonies was observed when the sample was coincubated with a negative control T-cell clone for HA-1H and no decrease in number of colonies was measured when clone 1–55 was coincubated with a bone marrow sample from another patient with CML who was negative for LB-ITGB2-1 (CML 1159). In conclusion, the data demonstrated that LB-ITGB2-1–specific T cells are capable of mediating specific cytolysis of malignant hematopoietic (progenitor) cells, further supporting the therapeutic value of LB-ITGB2-1 as target for immunotherapy to induce GVL reactivity after alloSCT without GVHD.

## Discussion

In this study, we identified two HLA-B*15:01–restricted MiHAs targeted by CD8 T cells in a CML patient who reached complete remission after alloSCT and DLI. T cells for these MiHAs showed strong recognition of patient CML-APC, but different reactivity against patient FB. Whereas clone 1–30 strongly recognized patient FB after pretreatment with IFNγ, clone 1–55 consistently failed to recognize (IFNγ pretreated) FB. We performed WGAS and identified SNP rs2275260 in the *GLE1* gene that encodes LB-GLE1-1V, the MiHA recognized by clone 1–30. For clone 1–55, associating SNP rs760462 in intron 3 of the *ITGB2* gene was found, but no SNP disparity could be detected in the normal *ITGB2* transcript between patient and donor. Here, we demonstrated that RNA-sequence analysis enabled discovery of LB-ITGB2-1 as MiHA encoded by an alternative transcript. LB-GLE1-1V and LB-ITGB2-1 have population frequencies of 35% and 21% in Caucasians (www.hapmap.org), resulting in disparity rates of 24% and 23%, respectively. LB-GLE1-1V and LB-ITGB2-1 are the first MiHA presented by HLA-B*15:01, which is expressed in approximately 7% of Caucasians (32). As such, these MiHAs may contribute to broaden immunotherapy to treat patients with hematologic malignancies after alloSCT.

As no SNP disparity was found in the normal *ITGB2* transcript, we investigated whether an alternative transcript could encode the MiHA. Alternative transcripts have previously been reported to encode ACC-6 (15) and ZAPHIR (16). ACC-6 is a MiHA that has been identified by screening a cDNA library, whereas ZAPHIR has been discovered by WGAS followed by cloning and screening transcript variants as detected by PCR. For *ITGB2*, we failed to detect splice variants by PCR using different primers. Retrospectively, this failure can be explained by absence of the forward or reverse primer binding site in the alternative *ITGB2* transcript. We therefore investigated *ITGB2* gene transcription by RNA-sequence analysis. In the GEUVADIS project, RNA-sequencing has been performed for EBV-B cell lines for which also whole genome data are available in the 1000 GP, allowing us to select samples for the

(*Continued.*) The allo-HLA-A*02:01–reactive T-cell clone HSS12 (38; right) was included as a control clone. Genotyping results (+ or −) for LB-ITGB2-1 (left), LB-GLE1-1V (middle), and HLA-A*02:01 (right) are indicated for the selected samples. Mean release of IFNγ of duplicate wells as measured by IFNγ ELISA after overnight coincubation of T cells and stimulator cells is depicted. B, lysis of patient CML-APC as well as other primary leukemic samples from HLA-B*15:01 patients who were positive for SNP rs760462 (LB-ITGB2-1) was measured in a 10-hour chromium-release assay. Clone 1–30 (LB-GLE1-1V), clone HSS12 (allo-HLA-A*02:01), and clone FK47 (HA-1H; ref. 39) were included as controls. Genotyping results (+ or −) for LB-ITGB2-1 (top left), LB-GLE1-1V (top right), HLA-A*02:01 (bottom left), and HA-1H (bottom right) are shown. Mean specific lysis of triplicate wells is depicted. C, lysis of CML progenitor cells from patient 6940 (PAT CML 6940) and patient 1159 (CML 1159) in a colony-forming assay. Peripheral blood or bone marrow samples were preincubated overnight in the absence or presence of T cells at an E:T ratio of 3:1. After overnight coincubation, single-cell suspensions were seeded and colony-forming units were enumerated for the granulocyte/macrophage (CFU-GM, CFU-G, CFU-M) and erythroid (CFU-E, BFU-E) lineages as well as for mixed granulocyte/macrophage/erythroid lineages. Indicated are the number of CFU for the LB-ITGB2-1 specific T-cell clone 1–55, the HA-1H–specific T-cell clone FK47 and the HLA-A*02:01–specific alloreactive T-cell clone HSS12. Patient 6940 is positive for LB-ITGB2-1 and HLA-A*02:01, but negative for HA-1H. Patient 1159 is positive for HLA-A*02:01, but negative for LB-ITGB2-1 and HA-1H. O/n indicates overnight coincubation.



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associating SNP as identified by WGAS. We demonstrated that associating SNP rs760462 functions as splice acceptor site, thereby creating an alternative transcript in which part of intron 3 is retained that encodes LB-ITGB2-1. We also demonstrated that antigens encoded by alternative transcripts that are generated by exon skipping can be elucidated by RNA-sequence analysis. As such, RNA-sequence analysis is a powerful tool to identify MiHAs, but its value will extend beyond the field of alloSCT, as neoantigens and other immune targets may also be encoded by alternative transcripts.

In human melanoma and small-cell lung carcinoma, tumor-specific mutations can create neoantigens. Neoantigens resemble minor histocompatibility antigens in that peptides are presented by HLA surface molecules and recognized by specific T cells (33). The chance that neoantigens are targeted after antibody or T-cell therapy is dependent on mutational load and tumors with <1 mutations per Mb coding DNA have been proposed to present neoantigens only occasionally. This prediction, however, is based on the presence of tumor-specific mutations in coding exons leading to single amino acid changes in the normal protein reading frame, whereas alternative splicing, a mechanism that is frequently deregulated in cancer (34, 35), has not been taken into consideration. However, when tumor-specific mutations in pre-mRNA sequences, spliceosomal components or regulatory factors lead to aberrant splicing, transcript variants can be produced that encode entirely new protein products. By producing these aberrant proteins, alternative splicing may create a repertoire of neoantigens that is larger than expected on the basis of mutational load. As alternative transcripts can be elucidated by RNA-sequence analysis, this technique may also be relevant to apply to cancer neoantigen discovery.

As T cells for LB-ITGB2-1 strongly recognized patient CML-APC, but lacked reactivity against patient FB, and *ITGB2* has been reported as gene with hematopoiesis-restricted expression, we investigated whether LB-ITGB2-1 may be a new MiHA with therapeutic relevance. We confirmed hematopoiesis-restricted expression of the normal *ITGB2* transcript by microarray gene expression analysis and demonstrated that the alternative *ITGB2* transcript followed the same pattern of expression by qPCR (Fig. 4). Therapeutic relevance of LB-ITGB2-1 was supported by recognition and lysis of leukemic samples of different origins by specific T cells. Only two CML samples, both expressing low levels of the alternative *ITGB2* transcript, were not recognized. One sample was obtained from our patient at diagnosis prior to alloSCT. This sample mainly consisted of mature myelocytes, which are poor stimulators of an immune response. Previous work in our laboratory demonstrated that leukemic APC can be generated from CD34-positive CML precursor cells as illustrated by detection of BCR-ABL (36). We therefore *in vitro* modified patient CML cells and used CML-APC to stimulate and isolate T cells after DLI. Our data demonstrated that CML-APC expressed increased levels of the alternative *ITGB2* transcript (Fig. 4B) and mediated specific T-cell recognition and lysis (Fig. 5). Thus, T cells for LB-ITGB2-1 as present in the DLI may have contributed to GVL reactivity *in vivo* by eliminating BCR-ABL positive CML precursor cells as detected during cytogenetic relapse after alloSCT. This is further substantiated by the finding that T cells for LB-ITGB2-1 are capable of mediating specific cytolysis of CML progenitor cells in a colony-forming assay. Moreover, T cells for LB-ITGB2-1 may have contributed to GVL reactivity by eliminating CML cells with an acquired APC phenotype *in vivo* either by coinfusion of IFNα, which is known to accelerate the GVL response (5, 6) or indirectly upon cytokine release by T cells with other specificities than LB-ITGB2-1. We previously demonstrated in a NOD/scid mouse model that human CML cells in lymphoid blast crisis can become professional APC after treatment with DLI (37). In our patient, T cells for three other MiHAs than LB-ITGB2-1 were detected after DLI (Supplementary Fig. S3), including LB-GLE1-V, which is strongly recognized on patient CML cells at diagnosis (Fig. 5A). As such, T cells for LB-ITGB2-1 may have cooperated with other immune cells in mediating the antitumor response. In contrast to patient CML cells at diagnosis, the majority of unmodified leukemic samples were directly recognized and lysed by clone 1–55, suggesting that in most patients, T cells for LB-ITGB2-1 are capable of mediating GVL reactivity independent of whether the leukemic cells become professional APC.

In our patient, GVL reactivity after DLI was accompanied with development of grade II skin GVHD. As *ITGB2* is not expressed in nonhematopoietic cell types and LB-ITGB2-1 could not be recognized on FB even after treatment with inflammatory cytokines, we consider it more likely that T cells with other specificities than LB-ITGB2-1 as measured in our patient after DLI (Supplementary Fig. S3) mediated or contributed to development of GVHD.

In summary, an integrated strategy of whole genome and transcriptome analysis enabled identification of LB-ITGB2-1 as HLA-B*15:01–restricted MiHAs encoded by an alternative transcript. The alternative *ITGB2* transcript was shown to be expressed in leukemic cells of different origins, whereas no expression was found in nonhematopoietic cell types from organs that are often targeted in GVHD. In addition, T cells specifically recognized and lysed leukemic cells of different origins, whereas no reactivity was measured against patient FB. As such, our data demonstrate the discovery of a new hematopoiesis-restricted MiHA with therapeutic value to augment GVL reactivity after alloSCT without GVHD and illustrate the relevance of RNA-sequence analysis to identify immune targets that are encoded by alternative transcripts and created by genetic variants.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** M.J. Pont, E.D. van der Meijden, J.H.F. Falkenburg, M. Griffioen

**Development of methodology:** M.J. Pont, E.D. van der Meijden, P.A.C. 't Hoen, J.H.F. Falkenburg, M. Griffioen

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** M.J. Pont, E.D. van der Meijden, M. Eefting, E.W.A. Marijt, J.H.F. Falkenburg, M. Griffioen

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** M.J. Pont, D.I. van der Lee, E.D. van der Meijden, C.A.M. van Bergen, M.W. Honders, M. Vermaat, S.M. Kielbasa, P.A.C. 't Hoen, J.H.F. Falkenburg, M. Griffioen

**Writing, review, and/or revision of the manuscript:** M.J. Pont, M. Eefting, E.W.A. Marijt, P.A.C. 't Hoen, J.H.F. Falkenburg, M. Griffioen

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Clinical Cancer Research

# Integrated Whole Genome and Transcriptome Analysis Identified a Therapeutic Minor Histocompatibility Antigen in a Splice Variant of *ITGB2*

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