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ORIGINAL ARTICLE

In situ analysis of the antigen-processing machinery in acute myeloid leukaemic blasts by tissue microarray

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Altered expression of major histocompatibility complex (MHC) class I molecules can be caused by defects in genes of the antigen-processing machinery (APM), and is often correlated to progression in solid tumours. However, little is known about expression of the APM components in blasts from patients with acute myeloid leukaemia (AML). In this study, we investigated the expression of the APM components large multifunctional peptidases (LMP) 2 and 7, transporter-associated with antigen processing (TAP) 1 and 2, β-2-microglobulin (β2m) and MHC class I heavy chain *in situ* by tissue microarray from bone marrow biopsies of 30 AML patients. APM components were heterogeneously expressed in all AML samples tested, but no significant correlation with the AML subtype according to the French-American-British classification was found. Depending on the APM component tested, up to 90% of the trephines showed no or weak expression, whereas the LMP7 protein was detected in 66% of all samples. By following disease progression in individual AML patients, we found severe downregulation of APM components in two out of four patients from initial diagnosis to relapse. We conclude that downregulation of APM components may play a role in the failure of immunosurveillance and may therefore contribute to relapse in acute leukaemia.

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Introduction

The antigen-processing machinery (APM) of the cell is a complex system of interacting proteins, responsible for presentation of peptides as major histocompatibility complex (MHC)-peptide complexes on the cell surface. This mechanism is crucial for recognition of virally infected cells, maintenance of self-tolerance and the surveillance of newly arising tumours by the immune system.[1] The presentation of tumour antigens on MHC class I surface molecules allows cytotoxic T lymphocytes to detect and, subsequently, kill antigen-specific malignant cells.[2,3] Defects in APM have been correlated with enhanced tumour growth and malignancy in solid tumours.[4–8] Furthermore, we and other groups have shown that the cytotoxic immune response can fail owing to altered antigen presentation by previous downregulation of APM components.[1,9,10]

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Surprisingly, little is known about the APM in haematopoietic malignancies, such as acute myeloid leukaemia (AML). Treatment of high-risk or relapsed AML patients is mainly based on the induction of a graft-versus-leukaemia effect after allogenic haematopoietic stem cell transplantation, where donor immune cells recognize minor histocompatibilty antigens on residual blasts and exert a cytotoxic response against leukaemia. Furthermore, there is growing evidence for a specific T-cell response against AML-associated antigens, such as adipophillin, survivin, mucin-1, proteinase-1, Wilms' tumour-1 and others.[11–14] In this study, we aimed to determine whether alterations in APM components are also a possible immune escape mechanism used by AML blasts to evade specific recognition and elimination by the immune system. We studied the APM at different levels of protein processing and assembly of the MHC-peptide complex. On the level of protein degradation, we evaluated the expression of the large multifunctional peptidases (LMPs) 2 and 7, which constitute parts of the immunoproteasome multiprotein complex. These particular peptidases are necessary for generating peptides that associate preferentially with the MHC class I molecules compared with peptides generated by the normal, constitutive proteasome.[15] The next step in antigen presentation is the transport of processed peptides to the endoplasmic reticulum, which is, among others, facilitated by the transporter-associated with antigen processing (TAP) 1 and 2.[16] Finally, peptides associate in the endoplasmic reticulum lumen with the MHC class I heavy chain (MHC class I HC) and β-2-microglobulin (β2m) to form a functional MHC-peptide complex presented on the cell surface.[17]

To investigate these APM components, we used a newly established method to assemble tissue microarrays (TMA) from bone marrow (BM) biopsies of AML patients.[18] The TMA technique was optimized for our purposes to enable us to preserve morphology to identify blasts precisely and to detect protein expression *in situ* on the cellular level. TMA offers the advantage of simultaneous evaluation of a large number of cases under standardized conditions making it a high-throughput and cost-effective technology. Using this technique, we were able, for the first time to characterize the APM component expression in AML cell lines and BM trephines from AML patients. Most interestingly, we could demonstrate downregulation of APM components in AML blasts obtained from individual AML patients at relapse compared with the first diagnosis.

Materials and methods

Patients

After informed consent, BM biopsies ($n=43$) from 35 patients with AML, which were enrolled in a clinical trial approved by

the institutional ethics committee, were analysed (for detailed information see Supplementary Table 1).

Cell lines

The cell lines K562, KG1a, U937, Daudi and 174x CEM.T2 (T2) were maintained in complete RPMI 1640 media (Biochrom, Berlin, Germany) supplemented with 10% (v/v) of fetal calf serum (PAA, Pasching, Austria). Fresh AML blasts from the peripheral blood (84% of blasts in peripheral blood) of patient P3 were isolated by density gradient centrifugation, and grown without further enrichment over several passages in complete media containing 10% (v/v) human AB Serum (PAN-Biotech, Aidenbach, Germany). To study the effect of interferon-γ (IFNγ) on the expression of APM components, aliquots of 10^6/ml P3 blasts or cell lines were incubated in the presence or absence of human recombinant 200 IU/ml IFNγ (PromoCell, Heidelberg, Germany) for 24 h. MHC class I expression in response to IFNγ treatment on cell lines and blasts from P3 was analysed by surface staining using PE-conjugated anti-HLA-A, B, C (clone G46-2.6, BD) and the matching isotype control by FACS (BD, FACS Canto). Data were analysed by FlowJo software (Tree Star). Additionally, blasts from P3 were used untreated or upon stimulation with 200 IU/ml IFNγ for TMA construction and RNA preparation.

Construction of TMA from BM biopsies and cell lines

Tissue microarray of BM biopsies was constructed in a manner similar to the method described previously.[18] Only BM biopsies with a clearly recognizable infiltration by blasts and well-preserved morphology were chosen for further processing. Areas of interest were marked on the haematoxylin and eosin-stained slide. Biopsies were taken from carefully preselected areas in the original paraffin blocks at defined array coordinates. We used a specially manufactured stainless steel tube with a wall thickness of 0.1 mm and a diameter of 1.6 mm. However, in TMAs constructed for this study, only one sample of the donor block was inserted into each hole of the acceptor block.

In addition, control-TMAs were mounted on the same slide as sections of the TMA of BM biopsies. These control-TMAs contained the above mentioned cell lines and the primary AML blasts of P3, as well as antigen-presenting cells. Antigen-presenting cells were generated as follows: monocytes from healthy donors were obtained by counter current elutriation and included in the TMA. Furthermore, monocytes were cultured in the presence of 500 IU/ml IL-4 (PromoCell), 500 IU/ml granulocyte–macrophage-colony-stimulating factor (Berlex, Seattle, WA, USA) and 5 ng/ml transforming growth factor-β (Peprotech, Rocky Hill, NJ, USA) for 7 days to generate immature dendritic cells. By the addition of 10 ng/ml IL-1β, 1000 IU/ml IL-6, 10 ng/ml tumour necrosis factor α (all PromoCell) and 1 μg/ml PG-E_2 (Pfizer, Karlsruhe, Germany) for the last 48 h, dendritic cells were terminally differentiated into mature dendritic cells, and included in the control-TMA as primary antigen-presenting cells. For construction of control-TMAs, cultured cells were fixed in formalin for 12 h, washed subsequently and the resulting cell pellet embedded into a paraffin block using the Shandon Cytoblock (Thermo Electron Corporation Anatomical Pathology, Pittsburgh, PA, USA).

Immunohistochemistry

The primary antibodies used in this study were LMP2 (clone SY-1), LMP7 (clone HB-2), TAP1 (clone NOB1), TAP2 (clone NOB2), β2m (clone NAMB-1) and MHC class I HC (clone HC-10). These antibodies have been used in previous studies of APM component expression in various tissues,[19,20] and were kindly provided by Dr S Ferrone (Roswell Park Memorial Institute, Buffalo, NY, USA). Staining for the different APM components was performed using standard immunohistochemical staining procedures with heat-induced antigen retrieval, and visualization of staining was performed using the avidin-biotin peroxidase method with diaminobenzidine chromatogen (LMP2, LMP7, MHC class I HC) or En-Vision (TAP1, TAP2, β2m; DakoCytomation, Glostrup, Denmark). TMAs of BM biopsies and control-TMAs were stained simultaneously to assure quality control; in most cases, sections of the control-TMAs were mounted onto the same slide as BM-TMAs.

Evaluation of TMAs

All tissue samples were evaluated without knowledge of clinical data by an experienced histopathologist (ECO). To make the immunohistochemical expression of the various markers in the neoplastic cells comparable, a semi-quantitative scoring system was used. First, neoplastic cells were identified and, subsequently, the percentage of positive cells in relation to all the blasts was estimated. APM protein expression was scored by the system established by Mehta *et al*.[6] in a similar experimental setting. If the percentage of positive blasts was 0, 1–5, 5–25, 25–50, 50–75% or above 75%, the score was 0, 1, 2, 3, 4 or 5, respectively. The intensity of the staining reflected the level of expression and was scored as 0 for no expression [−], 1 for very faint to weak [+], 2 for moderate [++] and 3 for strong [+++]. Both scores were added and the sum was used to classify expression of APM proteins into three categories, with a score of 7–8 reflecting high protein expression, a summed score of 3–6 showing partial expression of APM components and a total score of 2 or less being considered as faint expression. Individual TMA staining was done at least three times with the same result.

Enrichment of AML blasts by magnetic separation

Mononuclear cells from peripheral blood or BM were isolated by ficoll separation and stored for further use in fetal calf serum with 10% (v/v) dimethylsulphoxide (Merck, Darmstadt, Germany) in liquid nitrogen. After thawing the mononuclear cells gently, homogeneity of blast populations was tested by FACS analysis (BD, FACSCalibur) by surface expression of CD33, CD34, CD45 and CD133. Populations with more than 90% blasts were directly used in quantitative real-time PCR (qRT-PCR) analysis. Patient samples with lower frequencies of blasts were enriched by MACS magnetic bead positive selection for CD34 (Miltenyi, Bergisch Gladbach, Germany), according to the manufacturer's instructions. All samples used in this study had at least 90% blasts in the final fraction used for RNA isolation.

Preparation of nucleic acid and qRT-PCR

Total RNA was extracted from cell lines (Qiagen, Hilden, Germany) and cellular RNA was transcribed with Superscript II DNA-Polymerase (Invitrogen, Karlsruhe, Germany), according to the manufacturer's protocol. Quantitative PCR of cDNA was performed with the QuantiFast SYBR Green chemistry (Qiagen) on a Mastercycler realplex (Eppendorf, Hamburg, Germany) as two-step PCR (95 °C 5′; 95 °C 8″, 60 °C 20″; 40 cycles). All data

shown arose from at least two independent RNA preparations of different samples, each measured in duplicate.

As APM gene specific primers, the following oligo-DNAs were used (5′–3′): LMP2: GCATATAAGCCAGGCATGTCTCC and AGCTGTAATAGTGACCAGGTAGATGAC; LMP7: CGGGT GAACAAGGTGATTGAG and CCATTTCGCAGATAGTACAG CC; TAP1: CCAATATGAGCACCGCTACCT and CTGCAG CAGCTGTGATTTCC; TAP2: GCCCATCTCACAGTATGAACAC and CACCTTATCATCTTCGCAGCTC; β2m: CTCTTGTACTA CACTGAATTCACC and CAATGATGCTGCTTACATGTC. 18S rRNA was found to be the best housekeeping gene for internal standardization of target gene expression by the BestKeeper software.[21] 18S specific oligo-DNAs were (5′–3′): ACCGATTG GATGGTTTAGTGAG and CCTACGGAAACCTTGTTACGAC. The qRT-PCR experiments were repeated at least two times and analysed by the unpaired *t*-test.

Statistical analysis

Statistical analysis was performed by the GraphPad Prism5 software using the unpaired *t*-test for Figure 2c or the Mann–Whitney *U*-test (all other statistics), with results considered significant if $P<0.05$.

Results and discussion

Control-TMA for evaluation of the anti-APM antibodies

Conditions for APM-antibody staining were established with control-TMAs from cell lines with known defects in APM, such as the T-B-cell hybrid, T2, or the human Burkitt lymphoma-derived cell line, Daudi. T2 cells are negative for LMP2/7 and TAP1/2 owing to a large deletion of this locus on chromosome 6, whereas Daudi cells harbour a mutation in the *β2m* gene, which causes the abrogation of β2m protein expression.[22–24] Using the TMA technique, we confirmed the predicted phenotype of these cell lines (Figure 1a). As we wanted to study broadly expressed antigens, these controls were essential. Comparing expression levels of AML blasts simultaneously with negative controls could, therefore, validate faint or non-detectable expression of APM components owing to down-regulation on the protein level. As positive controls for correct TMA staining, different populations of antigen-presenting cells, such as monocytes and *in vitro* generated immature and mature dendritic cells from healthy donors, were used. These showed the expected high to very high expression of all APM components depending on their level of maturation (Supplementary Figure 1).[20]

We next evaluated the APM profile of different tumour cell lines, such as KG1a (AML), U937 (lymphoma cells with monocytic characteristic) and K562 (chronic myeloid leukaemia in blast crisis). K562 is a highly de-differentiated cell line that lacks MHC class I expression on the surface. Consequently, K562 cells showed a weak expression of the APM components LMP2 and TAP2, whereas LMP7, TAP1, β2m and MHC class I HC were completely negative. In contrast, U937 as well as KG1a cells showed a high expression of the majority of the APM proteins (Figure 1a).[25,26]

To confirm the relevance of the APM analysis by TMA, we next performed a comparative study using qRT-PCR. As shown in Figure 1b, qRT-PCR data correlated well with TMA data (Figure 1b), with the exception of β2m expression in Daudi cells. In these cells, low levels of β2m mRNA were detected, whereas no β2m protein was found in TMA staining. This is owing to a known G>C mutation in the ATG start-codon of the *β2m* gene, disrupting the protein coding the open reading frame of *β2m*, thus leading to a complete loss of β2m protein expression despite detectable β2m mRNA levels.[24]

Upregulation of the APM components in primary AML cells upon IFNγ exposure

We next asked whether the *in vitro* induction of the APM components in AML blasts can be quantified by TMA analysis. IFNγ is known to induce upregulation of APM and surface MHC class I and class II expression in different cell types.[27] Different cell lines and the primary AML blast cell line established from patient no. 3 (P3), were cultured for 24 h in the presence or absence of 200 IU/ml IFNγ. Aliquots of these cells were assessed for MHC class I surface expression by flow cytometry (Figure 2a). As expected, Daudi and K562 were negative for MHC class I expression, which could not be restored in the presence of 200 IU/ml IFNγ.[28,29] Expression of MHC class I in T2 cells was also not upregulated in response to IFNγ. Treatment of KG1a, U937 and blasts from patient P3 with IFNγ induced an increase in MHC class I expression, suggesting that regulatory mechanisms through IFNγ are functional in these lines. To examine a potential upregulation of particular APM components, we performed additional TMA and qRT-PCR analyses from primary blasts obtained from patient P3. The APM component expression was generally weak in untreated P3 AML blasts, as demonstrated in the TMA (Figure 2b), and confirmed the weak staining of MHC class I surface expression by FACS (Figure 2a). After IFNγ treatment, the expression of all APM components could be restored to significantly high levels within the blasts. The TMA data could be confirmed by qRT-PCR data, indicating a significant increase in mRNA expression after IFNγ exposure (Figure 2c).

APM expression profile in AML patients

Thirty-five patients included in this study and a total of 43 BM samples from these patients were selected for TMA construction. Finally, a total of 35 biopsies of 30 AML patients that yielded data within all six antibodies tested were selected for evaluation. These patients were classified into the following subtypes by the French-American-British classification system: M1 $n=5$, M2 $n=6$, M3 $n=1$, M4 $n=9$, M5 $n=5$ and not classified (NC) $n=5$ (detailed information in Supplementary Table 1).

In our study, we carefully pre-selected areas of interest in each trephine, and obtained a relatively large sample size of 1.6 mm diameter for each tissue core, which allowed us optimal assessment of the morphology. A considerably smaller sample size of 0.6 mm had been chosen by another group for the construction of BM-TMAs, who reported a failure rate of 11% in their immunohistochemical evaluation.[30]

As an example for the expression of APM components tested, individual TMAs with their respective expression scores are given in Figure 3a. Semiquantitative analyses of all APM components tested are summarized in Figure 3b. Evaluation of LMP2, LMP7, TAP1, TAP2, β2m and MHC class I HC expression showed a heterogeneous expression of all APM components. A high level of MHC class I HC expression was detected in all biopsies tested with a median score of 8 and was, therefore, not a subject of further comparative analysis. The expression of all other APM components was either not detectable or only partially detectable in the majority of AML blasts (70–90%, Table 1), with the exception of LMP7, which was expressed in the majority (66%) of all AML samples. Of interest, blasts from all AML patients failed to express at least one single APM protein

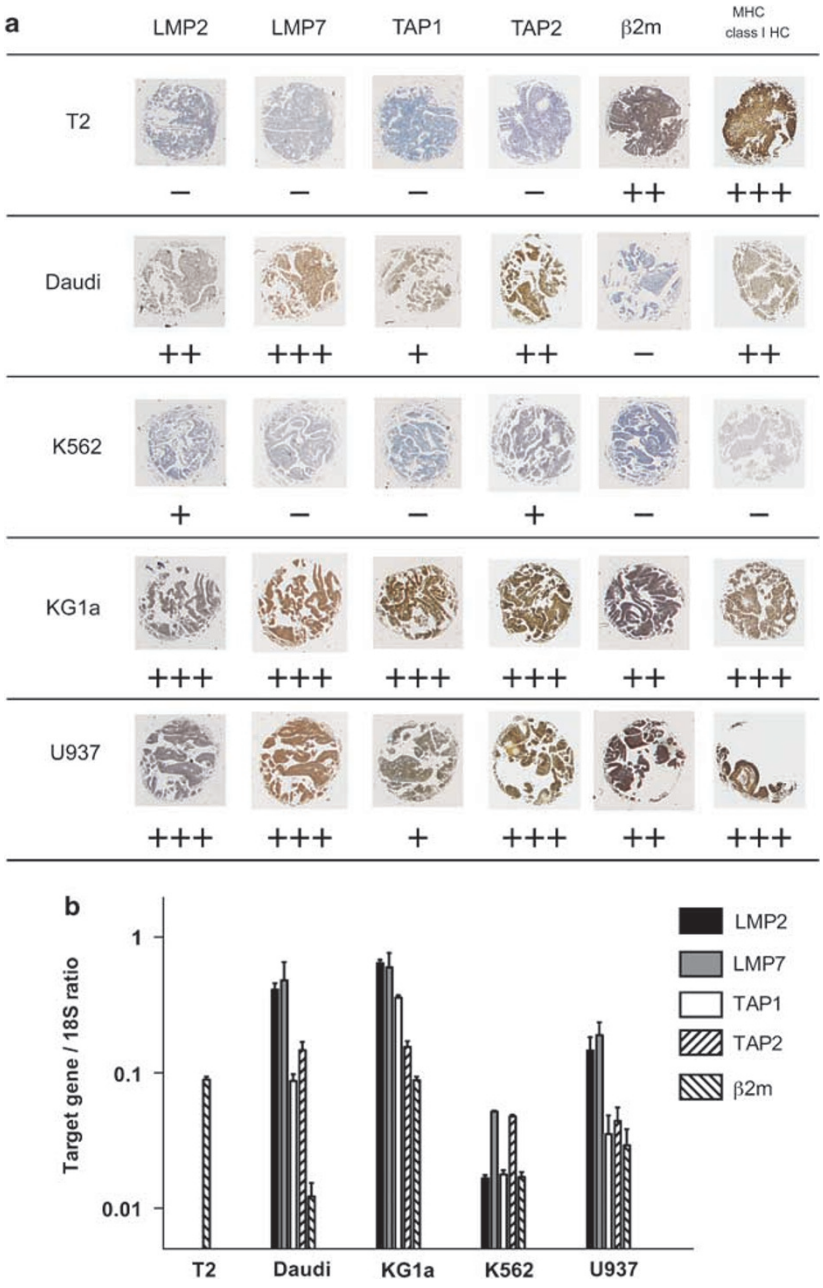

Figure 1 Expression of antigen-processing machinery (APM) components in different haematopoietic tumour cell lines. (**a**) A control panel of cell lines (T2, Daudi, K562, KG1a and U937) were spotted and stained with the anti-APM monoclonal antibodies specific for LMP2, LMP7, TAP1, TAP2, β2m and MHC class I HC. Intensity of staining in these cells was classified as negative [−], faint [+], moderate [++] or strong [+++]. (**b**) Expression of mRNA for the APM components LMP2, LMP7, TAP1, TAP2 and β2m were detected by quantitative real-time PCR and given as ratio to the housekeeping gene 18S RNA.

(Table 2), whereas the vast majority of samples (94%) showed multiple (two or more) APM expression defects, and one-third (34%) of all blasts lacked any expression of all APM components tested (Table 2).

Next, blasts obtained from peripheral blood or BM aspirates from seven patients out of the cohort were studied for the APM expression at the mRNA level. For this purpose, we enriched CD34+ AML blasts by magnetic separation. Even though blast populations of a purity of 90–95% could be obtained, we were unable to confirm the TMA data with qRT-PCR data (Supplementary Figure 2). Despite regulatory mechanisms on the translational level, this difference may be owing to the remaining 5–10% contaminating, non-malignant cells, which made the qRT-PCR data uninterpretable. As a common and reliable marker for AML blasts has not been found to date, enrichment to higher purities is difficult and hence would have to be tested for each patient individually. Additionally, blast populations can comprise different subclones, which are only distinguishable by histopathology or flow cytometric analysis. The TMA technique, as presented here, offers the unique advantage of not yielding a single, sometimes arbitrary, discriminator, but relies on the experienced eye of

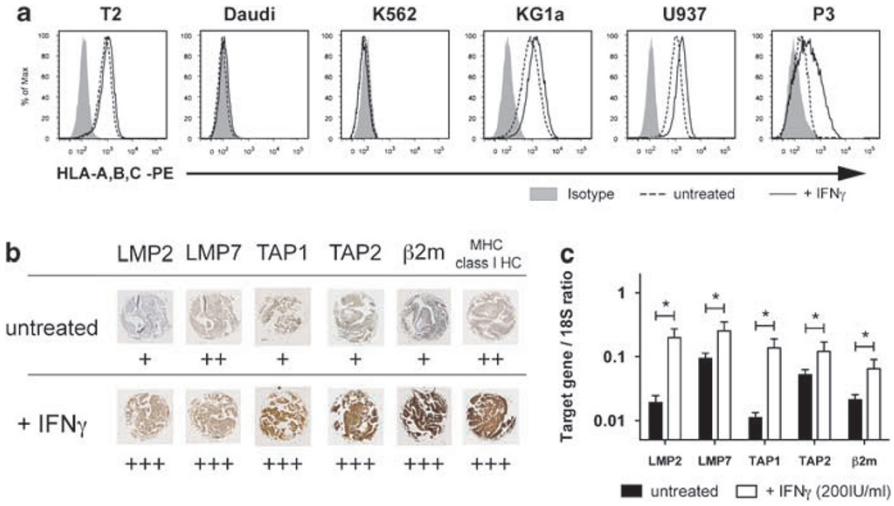

Figure 2 Upregulation of antigen-processing machinery (APM) components in primary acute myeloid leukaemia (AML) blasts after interferon-γ (IFNγ) exposure. (**a**) The cell lines, T2, Daudi, K562, KG1a, U937 as well as the primary AML blast line from patient P3 were incubated in the presence or absence of 200 IU/ml IFNγ for 24 h. Surface expression of MHC class I (HLA-A, B, C) was analysed by flow cytometry. As isotype controls of untreated and IFNγ-treated samples showed no difference, only isotype controls of IFNγ-treated cells are shown (grey profile: isotype, dashed line: untreated; solid line: + IFNγ). Additionally, blasts from AML patient P3 were incubated for 24 h in the presence or absence of human recombinant IFNγ (200 IU/ml) and analysed for the expression of APM proteins (**b**) and mRNA (**c**). (**b**) Intensity of staining within the blasts was classified as negative [−], faint [+], moderate [++] or strong [+++]. (**c**) Expression of mRNA for APM components was detected by quantitative real-time PCR and given as ratio to the housekeeping gene 18S RNA.

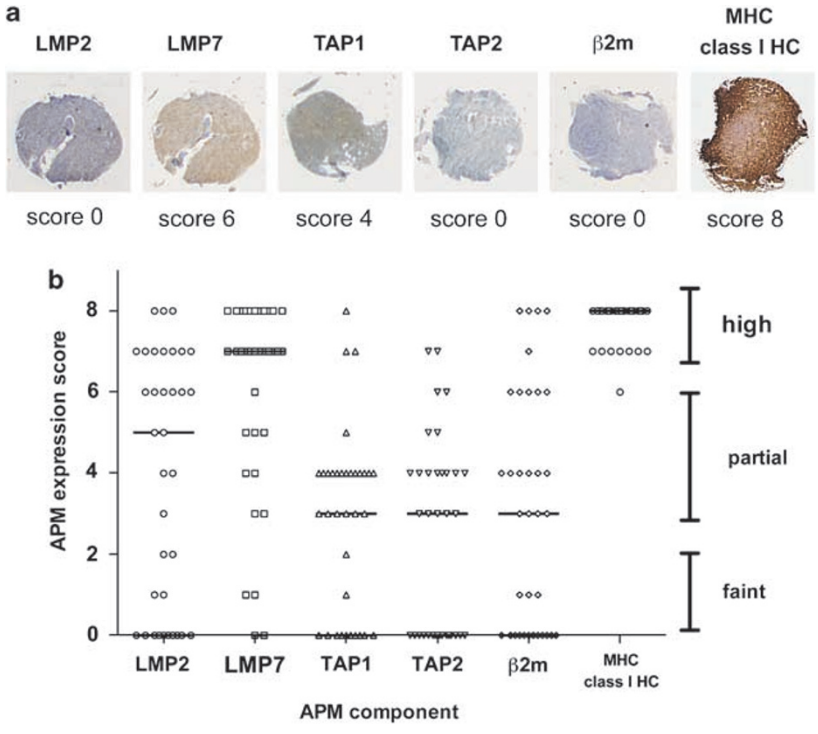

Figure 3 Expression of antigen-processing machinery (APM) components in acute myeloid leukaemia (AML) patients assessed by tissue microarray (TMA). (**a**) Individual TMA staining of all APM components analysed is shown as an example. Finally, 35 biopsies from 30 AML patients were accessible for analysis of expression of all APM components evaluated in this study. (**b**) Summary of TMA staining for APM components LMP2, LMP7, TAP1, TAP2, β2m and MHC class I HC using the expression score as described in the 'Materials and methods' section. Medians are indicated by black bars for each APM component.

the analysing pathologist. The identification of blasts within the background of normal haematopoietic cells allows the individual evaluation not only of single patients but also down to the cellular level.

Next, we investigated a possible association of TMA-derived APM expression data and the French-American-British classification, but were unable to point out any significant differences (Figure 4). Notably, blasts from AML M4 patients showed two

Table 1 APM components in BM biopsies of AML patients

Expression	*LMP2*	*LMP7*	*TAP1*	*TAP2*	*β2m*
High	10 (29%)	23 (66%)	3 (9%)	2 (6%)	5 (14%)
Partial	11 (31%)	8 (23%)	20 (57%)	17 (49%)	15 (43%)
Faint	14 (40%)	4 (11%)	12 (34%)	16 (46%)	15 (43%)

Abbreviations: AML, acute myeloid leukaemia; APM, antigen-processing machinery; BM, bone marrow.
A total of 35 BM biopsies from 30 AML patients were analysed for expression of the APM components LMP2, LMP7, TAP1, TAP2 and β2m by TMA. Percentages of AML blasts and intensity of expression were combined in an expression score and classified, as described in 'Materials and methods' section. Numbers and frequencies are given for the various APM components.

clearly distinct groups in terms of LMP2 and LMP7 expression, with either totally non-detectable expression (expression score 0) or high expression (score $\geqslant 6$) most pronounced for LMP2, but no M4 AML blasts with an intermediate LMP2 expression could be detected.

We next asked whether the combined defects of LMP2 and LMP7 protein expression as indicators for inducible immunoproteasome proteins, as well as combined defects of the transporter subunits TAP1 and TAP2, are detectable in AML patients. In 14% of the blasts, a faint expression of both LMP2 and LMP7 could be detected, whereas a lack of expression of TAP1 and TAP2 was more frequently found (31%, Table 3). The co-expression of LMP2 together with TAP1 was investigated, as the genes of these two proteins share a bi-directional promoter,

Table 2 Lack of multiple APM components in AML blasts

Number of APM components missing	*0*	*1*	*2*	*3*	*4*	*5*
Number of biopsies	0 (0%)	2 (6%)	4 (11%)	6 (17%)	11 (31%)	12 (34%)

Abbreviations: AML, acute myeloid leukaemia; APM, antigen-processing machinery.
Shown is the sum of patients lacking no (0), one (1) or up to all (5) APM components investigated. Numbers and frequencies are given for loss of the various APM components.

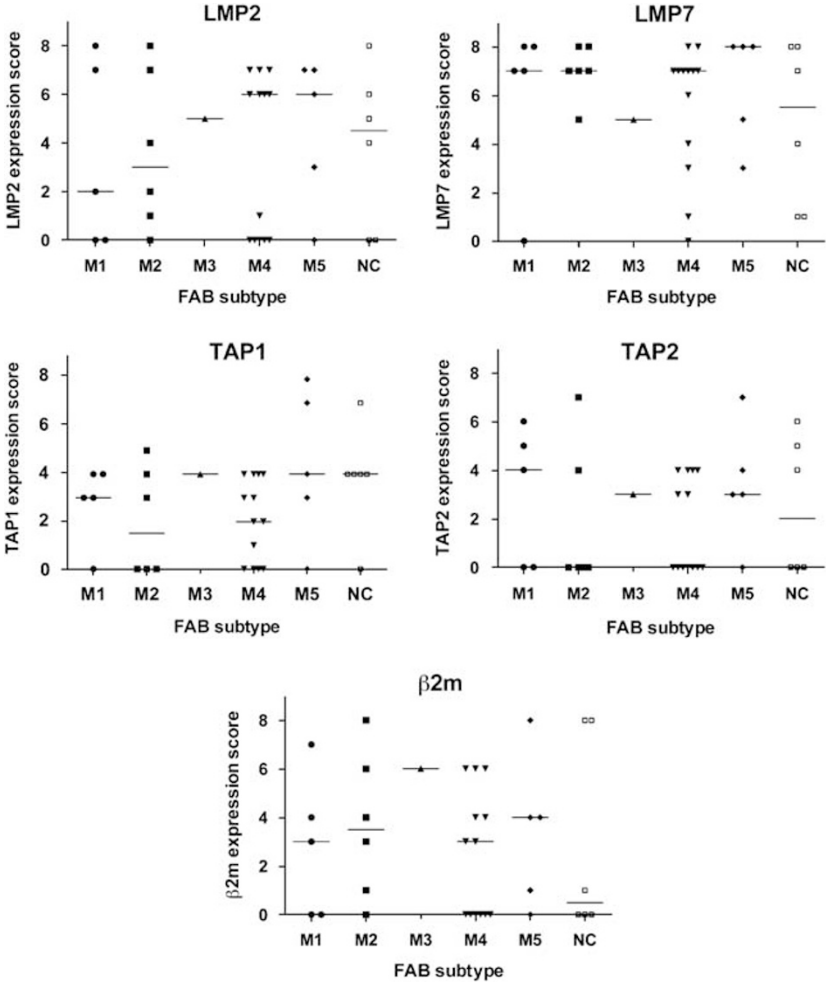

Figure 4 Acute myeloid leukaemia (AML) French-American-British (FAB) subtypes and expression score of antigen-processing machinery (APM) components. The expression score of the APM components LMP2, LMP7, TAP1, TAP2 and β2m are given for the different AML FAB-subtypes. NC, not classified.

which is thought to regulate divergent transcription.[31] The combined low expression of both proteins was found in 25% of the AML samples (Table 3).

Mechanistic studies have shown that the subunits LMP2 and LMP7 are not required for the generation of peptides in general.[32] These subunits appear to be crucial for the generation of peptides with hydrophobic and basic C-terminal residues,[15] which have been shown to bind preferentially to TAP molecules for the translocation into the endoplasmic reticulum lumen.[33] AML blasts lacking the LMP2 and LMP7 proteasomal subunits are, therefore, limited in their ability to process immunogenic proteins to peptides presented on MHC-peptide complexes. The combined lack of expression for TAP1 and TAP2 is even more severe, as only the heteromeric transporter, comprising both subunits, can facilitate the peptide transport into the endoplasmic reticulum lumen.[16] Recent *in vitro* studies have showed that TAP1 is needed to stabilize newly synthesized TAP2,[34] which could explain the high percentage of AML blasts lacking both TAP1 and TAP2 proteins. If there is no or only a minor TAP1 expression, the threshold for stabilization of TAP2 might be too low to be detected as well. Only one patient was found to express high levels of both subunits (Table 3).

Table 3 Combined defects in APM expression

	Faint expression	*Partial expression*	*High expression*
LMP2+LMP7	5 (14%)	13 (36%)	10 (28%)
TAP1+TAP2	11 (31%)	32 (89%)	1 (3%)
LMP2+TAP1	9 (25%)	24 (67%)	1 (3%)

Abbreviation: APM, antigen-processing machinery.
Numbers and frequencies of patients, which were classified with combined defects in AMP component expression, are given for the various combinations (see text). Patients with differential expression in these combinations were excluded from analysis.

Taken together, the APM data presented here show multiple deficiencies in APM protein expression in the AML blasts, which could lead to an escape from immuno-surveillance and, finally, may contribute to relapsing disease in these patients. The mechanisms underlying differential expression of LMP2/LMP7, TAP1/TAP2 and β2m in AML patients are, probably due to the heterogeneity of the disease, diverse. These may include mutation, deletion or loss of heterozygosity of APM genes, as well as epigenetic mechanisms.[35,36] Treatment of AML with demethylating agents (for example, decitabine) and inhibitors of histone deacetylases (for example, valpronic acid) show potent antileukaemic effects.[37] In addition, these drugs have been shown to induce upregulation of APM components in various models,[38–40] which makes them an attractive tool to study mechanisms of APM dysregulation in AML.

APM expression in AML progression

The most interesting question in our study was the evaluation of trephines of individual AML patients who were refractory to induction chemotherapy or developed a relapse after chemotherapy. As BM biopsies were mostly taken at initial diagnosis (ID), only four pairs of corresponding biopsies from the same patient could be obtained taken at ID and relapse (Figure 5). Figure 5a shows a comparison of staining for LMP7 and TAP1 of two individual patients, P4 and P18, at ID and relapse. Although the expression score of LMP7 in P4 was only mildly reduced at relapse, P18 showed a complete downregulation of the proteasome subunit. TAP1 expression was slightly expressed in both patients at ID and completely lost after induction therapy. We summarized the expression scores for all patients with the corresponding biopsies in Figure 5b.

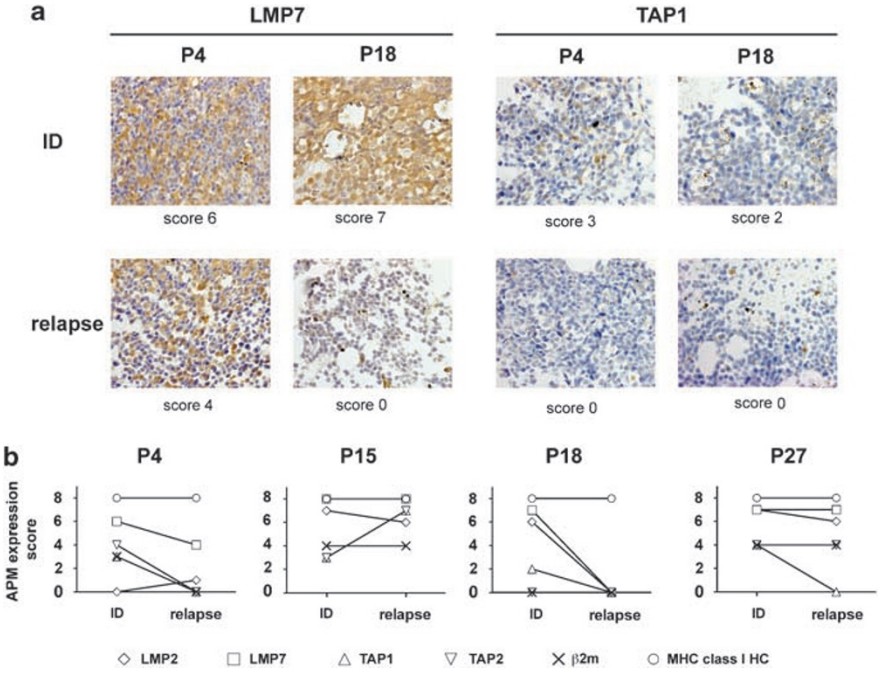

Figure 5 Downregulation of antigen-processing machinery (APM) expression in acute myeloid leukaemia (AML) relapse. (**a**) Individual tissue microarray (TMA) staining for LMP7 and TAP1 of patients P4 and P18 at initial diagnosis (ID) as well as after relapse are depicted (magnification 40 ×). Scores are given below each picture according to the scoring system described in 'Materials and methods' section. (**b**) Bone marrow trephines of four different AML patients were obtained from initial diagnosis (ID) as well as after relapse and were analysed for APM expression by TMA.

Interestingly, patients P4 and P18 (both AML M4) showed severe downregulation with an almost complete loss of APM protein expression at the time of relapse after high-dose cytarabin and antracyclin-based induction and consolidation chemotherapy, whereas patients P15 (AML M5) and P27 (AML M4) showed no or only little variation in APM expression throughout AML progression after chemotherapy (P27) or allogeneic peripheral blood stem cell transplantation (P15, Figure 5b). By following the APM component expression in disease progression in patients P4 and P18, we showed here, for the first time, that APM components can be completely downregulated in AML relapse. Therefore, the presentation of peptide-MHC class I complexes with more immunogenic peptides could be defective and could result in the failure of cytotoxic T lymphocyte-mediated cytotoxicity against AML blasts. In these cases, the selective pressure on APM components could be because of induction and consolidation chemotherapy. Studies by Wetzler *et al*.[20] showed that general MHC class I downregulation on AML blasts could not be detected in patients from ID to relapse, confirming our data on persistent high expression of MHC class I HC from ID to relapse in all four patients evaluated (Figure 5b). However, from our studies presented here, we conclude that the pattern of peptides presented on the MHC class I of AML blasts can change in disease progression and can, therefore, promote escape from the immune system. Hence, a larger cohort of paired samples taken at ID and relapsing disease will help to show the importance of APM expression and disease outcome in AML. Furthermore, we will analyse BM biopsies of AML patients for other components of the complex APM system, such as MECL10, PA28, Tapasin and ERp57 by TMA.

Conclusion

In summary, we demonstrate here, for the first time, that changes in the APM may play an important role in the progression of AML using the newly developed technique of TMA construction from BM trephines. Downregulation of APM components can contribute to immune escape of residual blasts after chemotherapy and can, therefore, promote relapse and disease progression in these patients.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)