ORIGINAL ARTICLE

Immune Escape of Relapsed AML Cells after Allogeneic Transplantation

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ABSTRACT

BACKGROUND
As consolidation therapy for acute myeloid leukemia (AML), allogeneic hematopoietic stem-cell transplantation provides a benefit in part by means of an immune-mediated graft-versus-leukemia effect. We hypothesized that the immune-mediated selective pressure imposed by allogeneic transplantation may cause distinct patterns of tumor evolution in relapsed disease.

METHODS
We performed enhanced exome sequencing on paired samples obtained at initial presentation with AML and at relapse from 15 patients who had a relapse after hematopoietic stem-cell transplantation (with transplants from an HLA-matched sibling, HLA-matched unrelated donor, or HLA-mismatched unrelated donor) and from 20 patients who had a relapse after chemotherapy. We performed RNA sequencing and flow cytometry on a subgroup of these samples and on additional samples for validation.

RESULTS
On exome sequencing, the spectrum of gained and lost mutations observed with relapse after transplantation was similar to the spectrum observed with relapse after chemotherapy. Specifically, relapse after transplantation was not associated with the acquisition of previously unknown AML-specific mutations or structural variations in immune-related genes. In contrast, RNA sequencing of samples obtained at relapse after transplantation revealed dysregulation of pathways involved in adaptive and innate immunity, including down-regulation of major histocompatibility complex (MHC) class II genes (*HLA-DPA1*, *HLA-DPB1*, *HLA-DQB1*, and *HLA-DRB1*) to levels that were 3 to 12 times lower than the levels seen in paired samples obtained at presentation. Flow cytometry and immunohistochemical analysis confirmed decreased expression of MHC class II at relapse in 17 of 34 patients who had a relapse after transplantation. Evidence suggested that interferon-γ treatment could rapidly reverse this phenotype in AML blasts in vitro.

CONCLUSIONS
AML relapse after transplantation was not associated with the acquisition of relapse-specific mutations in immune-related genes. However, it was associated with dysregulation of pathways that may influence immune function, including down-regulation of MHC class II genes, which are involved in antigen presentation. These epigenetic changes may be reversible with appropriate therapy. (Funded by the National Cancer Institute and others.)

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Most patients with acute myeloid leukemia (AML) ultimately have a relapse and die from progressive disease, despite initial sensitivity to chemotherapy. For this reason, patients who are in complete remission generally receive consolidation treatment with either additional chemotherapy or allogeneic hematopoietic stem-cell transplantation, a therapy that is thought to provide a benefit in part by means of an immune-mediated graft-versus-leukemia effect.[1-3] Although allogeneic transplantation is an effective therapy for patients with AML, relapse after transplantation is common and is associated with particularly poor outcomes.[4,5]

At relapse, AML cells often develop chromosomal gains and losses, and this finding has long suggested that therapeutic selective pressure can cause clonal evolution.[6-8] Our group and others have reported that AML relapse after chemotherapy has often been associated with gains and losses of subclones that contain unique somatic mutations, including putative driver mutations.[9-13] Recent studies that investigated the clonal evolution associated with AML relapse after transplantation were focused on recurrently mutated AML genes; although the presence of certain mutations can be used to predict an increased risk of relapse,[14-16] the mechanisms by which these mutations promote relapse remain unclear.

Previous studies showed down-regulation or inactivation of major histocompatibility complex (MHC) genes in AML cells at the time of relapse.[17-20] MHC genes have a critical role in antigen presentation and stimulation of antitumor immune responses, and their loss in post-transplantation relapse is one clear-cut mechanism by which relapsing tumors can escape immune surveillance. In patients who have received a transplant from a haploidentical donor, elimination of the mismatched HLA allele can occur; this event is uncommon in HLA-matched transplantation.[20,21] In this study, we performed a comprehensive analysis of samples from patients who had a relapse of AML after transplantation to define the genetic and epigenetic alterations that allow leukemic cells to escape the graft-versus-leukemia effect and to determine whether the dysregulation of known immune-related genes is a common feature of relapse after transplantation.

Methods

Patients

Samples were obtained as part of a study that was approved by the Human Research Protection Office at Washington University School of Medicine. All the patients provided written informed consent that permitted whole-genome sequencing, in accordance with a protocol that was approved by the institutional review board at the Washington University School of Medicine. For the discovery group, we identified patients who had adequate banked samples that were obtained at initial presentation with AML and at relapse; 15 adult patients had a relapse of AML after allogeneic hematopoietic stem-cell transplantation, and 20 adult patients had a relapse of AML after chemotherapy (14 of whom were included in a previous study[22]). To validate changes in MHC class II expression on flow cytometry or immunohistochemical analysis, additional samples from 28 patients who had a relapse of AML after transplantation were analyzed.

Molecular Analyses

DNA and RNA were isolated from samples of cryopreserved patient bone marrow. Samples with a low percentage of AML blasts were flow-sorted to enrich the blast population before the isolation of DNA or RNA. Control samples of skin or purified T cells (in two patients) were also sequenced, which allowed us to define variants as somatically acquired. Immunohistochemical analysis for HLA-DR was performed by NeoGenomics Laboratories and interpreted by a board-certified hematopathologist. Further details regarding flow cytometry, cell culture, and bioinformatic techniques are provided in the Methods section in the Supplementary Appendix, available with the full text of this article at NEJM.org.

Statistical Analysis

On the basis of the binomial probability distribution and a sample of 15 patients who had a relapse after transplantation, we used the Stat Trek calculator (https://stattrek.com/online-calculator/binomial.aspx) to calculate the likelihood of detecting a previously unknown relapse-specific mutation. If such a mutation were to have a true prevalence of 50% among all patients with a post-transplantation relapse, then the probability

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that we would observe the mutation in at least 3 of the 15 patients would be more than 90%. Further details on the statistical analysis are provided in the Methods section in the Supplementary Appendix.

RESULTS

ANALYSIS FOR RELAPSE-SPECIFIC MUTATIONS

To determine whether AML relapse after transplantation was associated with recurrent mutations, we performed enhanced exome sequencing on samples from 15 patients who had a relapse after transplantation, including 6 patients who presented with isolated extramedullary disease. The transplants had been from HLA-matched related donors, HLA-matched unrelated donors, or HLA-mismatched unrelated donors; no transplants had been from haploidentical donors (Table S1 in the Supplementary Appendix). Four of the patients with a post-transplantation relapse had had a post-chemotherapy relapse before they had undergone transplantation, and samples that had been obtained at the time of the post-chemotherapy relapse were also sequenced. For comparison, we evaluated 20 patients who had a relapse after chemotherapy alone. Among the patients in the two groups, there was a typical range in clinical variables, including donor type, time to relapse, and the use of immunosuppression at the time of relapse (Table S1 and Table S2 in the Supplementary Appendix).

Figure S1 in the Supplementary Appendix shows the status of 82 genes that were mutated in at least two samples from either group. Although more than 250 genes were mutated exclusively in patients with a post-transplantation relapse (Table S3 in the Supplementary Appendix), only 2 genes (*ETV6* and *FAM98B*) were mutated in more than one patient, and each of these 2 genes was mutated in only two patients. Thus, no driver mutations were commonly associated with relapse after transplantation among these patients, with the caveat that our sample would allow us to detect only a previously unknown relapse-specific driver mutation with a true prevalence of at least 50% among all patients with a post-transplantation relapse. In general, the recurrent mutations that were found at relapse after transplantation were similar to the mutations that were found at initial presentation and at relapse after chemotherapy, and no relapse-specific mutational patterns were observed.

ANALYSIS FOR SOMATIC MUTATIONS AND STRUCTURAL VARIANTS IN IMMUNE-RELATED GENES

Given the importance of the graft-versus-leukemia effect in the clearance of AML cells, we hypothesized that AML cells that recur after transplantation may have mutations that lead to immune escape.[23,24] Among the 15 patients with a post-transplantation relapse, we found no relapse-specific mutations in genes involved in antigen presentation, cytokine signaling, or immune-checkpoint modulation. Gene amplifications in *PDL1* and *PDL2* have been implicated in immune escape in Hodgkin's lymphoma.[25] In our study, only 1 of the 15 patients (Patient 814916) had an amplification in this region (Fig. S2 and Table S4 in the Supplementary Appendix), a finding that suggests that this is not a common mechanism of immune escape in AML after transplantation. We did not find any recurrent, relapse-specific structural variants in any region of the relapse genomes. Also, we did not identify any relapse-specific gene-fusion events; specifically, we found no fusions involving the MHC class II regulatory gene *CIITA* (Table S5 in the Supplementary Appendix).[26,27] In a previous study involving patients who had a relapse of AML after receiving a transplant from a haploidentical donor, loss of the mismatched HLA locus was identified in 5 of 17 patients.[20] In our study, only 1 of the 15 patients (Patient 113971) had a deletion at this locus (Fig. S2 in the Supplementary Appendix), and the deletion did not involve a coding region. None of the 3 patients who had received a transplant from an HLA-mismatched unrelated donor (Patients 312451, 440422, and 866660) had detectable deletions or mutations in mismatched HLA genes. In addition, analysis of single-nucleotide polymorphisms revealed no evidence of a copy-neutral loss of heterozygosity (i.e., uniparental disomy) in this region for any patient (data not shown).

ANALYSIS FOR CHANGES IN EXPRESSION OF IMMUNE-RELATED GENES

Since mutations in known immune-related genes were not observed in AML relapse after transplantation, we speculated that epigenetic changes in AML cells might have a role in disease progres-

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sion. We therefore performed total RNA sequencing on enriched AML blasts from paired samples obtained at initial presentation and at relapse from seven patients with a post-transplantation relapse and from nine patients with a post-chemotherapy relapse who had adequate cryopreserved material for flow-sorting. In each group, we identified genes that had significantly altered expression in relapse samples, as compared with presentation samples. Among the patients with a post-chemotherapy relapse, there were only 8 genes with a change in expression that met the prespecified cutoff for significance (false discovery rate, <0.05) (Table S6 and Table S7 in the Supplementary Appendix). In contrast, among the patients with a post-transplantation relapse, 34 genes were significantly up-regulated and 187 genes were significantly down-regulated (Table S6 in the Supplementary Appendix).

We performed an analysis of the pathways of genes with differential expression between presentation and relapse samples from the patients with a post-transplantation relapse. Significantly dysregulated pathways included pathways involving immune-response genes ($P<1\times10^{-35}$ for the enrichment of previously annotated immune-response genes among differentially expressed genes identified in this analysis) (see the Methods section in the Supplementary Appendix), as well as pathways involved in cell adhesion and motility and in the innate immune response (Table S8 in the Supplementary Appendix). Of the 30 most highly enriched pathways that involved "biological processes" (as designated by the Gene Ontology Consortium), 3 were related to cell adhesion, 6 to the innate immune response, and 13 to the adaptive immune response (Fig. 1, and Figs. S3 and S4 in the Supplementary Appendix).

Although the means by which these dysregulated genes may affect relapse after transplantation is not yet clear, the down-regulation of MHC class II genes suggests a plausible mechanism that may contribute to immune escape after transplantation; we therefore chose to focus on these changes. Four classical MHC class II genes (*HLA-DPA1*, *HLA-DPB1*, *HLA-DQB1*, and *HLA-DRB1*) were significantly down-regulated in six of the seven patients with a post-transplantation relapse. In addition, expression of the *HLA-DQA1*, *HLA-DRB3*, and *HLA-DRA* genes was decreased, but the changes were not significant (Fig. 1, and Fig. S4 in the Supplementary Appendix). Several other genes involved in antigen processing and presentation by MHC class II molecules (e.g., *IFI30*, *HLA-DMA*, *HLA-DMB*, and *CD74*) were significantly down-regulated in the same six patients, as was the gene encoding the T-cell costimulatory molecule CD86 (also known as B7-2 or CD28 ligand 2) (Fig. 1, and Fig. S4 in the Supplementary Appendix). The AML cells from one patient (Patient 440422) did not show down-regulation in any of these genes, which suggests that other mechanisms of relapse after transplantation must also be relevant for some patients.

To confirm the down-regulation of MHC class II genes at the protein level, we performed flow cytometry on paired presentation and relapse samples from six patients. Samples from all but one patient (Patient 619751) showed concordance between the down-regulation of MHC class II genes on RNA sequencing and the results on flow cytometry, which was performed with the use of a panspecific antibody for HLA-DP, HLA-DQ, and HLA-DR, with gating on the AML blast population (CD45 dim, side scatter low) (Fig. 2A, and Fig. S5 in the Supplementary Appendix).

RNA expression of MHC class I genes was decreased in some relapse samples, but the changes were not significant (Fig. S4 in the Supplementary Appendix). No consistent changes in the expression of MHC class I proteins were detected on flow cytometry of AML cells (Fig. 2A, and Fig. S6 in the Supplementary Appendix). Furthermore, other genes that have been proposed to have a role in immune tolerance in cancer (*PDL1*, *PDL2*, and *IDO1*) had low or no detectable expression in AML cells, a finding that suggests that these genes are either not important for relapse after transplantation or not regulated at a transcriptional level (Fig. S4A and Table S6 in the Supplementary Appendix).

VALIDATION OF DOWN-REGULATION OF MHC CLASS II

To determine the prevalence of down-regulation of MHC class II in a larger group of patients with relapse of AML after transplantation, we identified additional patients with samples in our tissue repository for whom cryopreserved paired presentation and relapse samples were available. Samples for 10 additional patients with a post-transplantation relapse were identified and analyzed with flow cytometry, yielding a total of 16 patients with a post-transplantation relapse (including the original patients). All the presenta-

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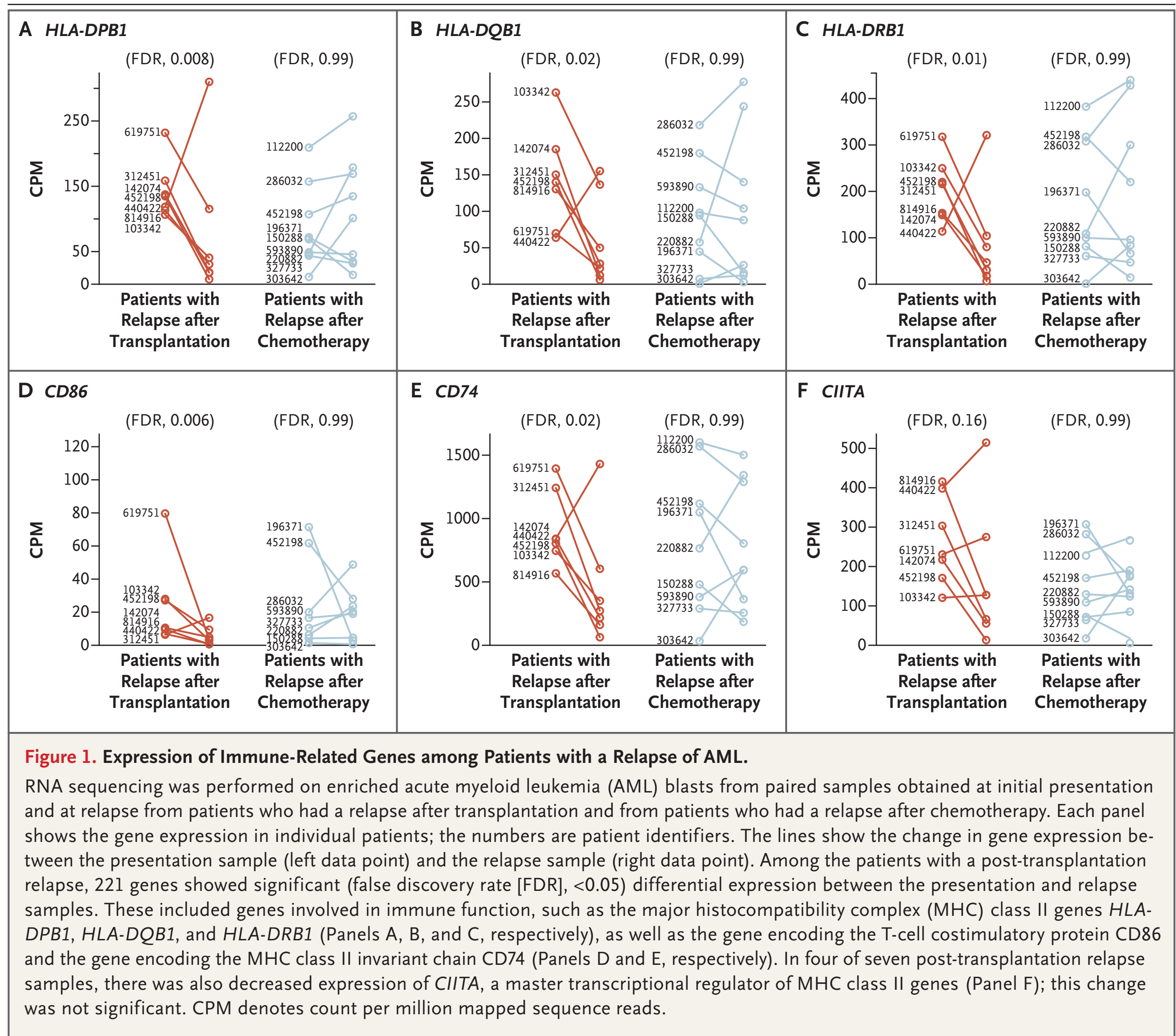

Figure 1. Expression of Immune-Related Genes among Patients with a Relapse of AML.

RNA sequencing was performed on enriched acute myeloid leukemia (AML) blasts from paired samples obtained at initial presentation and at relapse from patients who had a relapse after transplantation and from patients who had a relapse after chemotherapy. Each panel shows the gene expression in individual patients; the numbers are patient identifiers. The lines show the change in gene expression between the presentation sample (left data point) and the relapse sample (right data point). Among the patients with a post-transplantation relapse, 221 genes showed significant (false discovery rate [FDR], <0.05) differential expression between the presentation and relapse samples. These included genes involved in immune function, such as the major histocompatibility complex (MHC) class II genes *HLA-DPB1*, *HLA-DQB1*, and *HLA-DRB1* (Panels A, B, and C, respectively), as well as the gene encoding the T-cell costimulatory protein CD86 and the gene encoding the MHC class II invariant chain CD74 (Panels D and E, respectively). In four of seven post-transplantation relapse samples, there was also decreased expression of *CIITA*, a master transcriptional regulator of MHC class II genes (Panel F); this change was not significant. CPM denotes count per million mapped sequence reads.

tion samples showed high expression of MHC class II proteins, a finding that is consistent with results that have been reported previously (Fig. S5 in the Supplementary Appendix).[28,29] In 5 of the 16 post-transplantation relapse samples, MHC class II protein levels were at least 60 times lower than the levels seen in the paired presentation samples (as measured by the decrease in mean fluorescence intensity); the decreased levels were similar to the levels seen in negative controls. An additional 3 relapse samples had MHC class II protein levels that were 4 to 22 times lower than the levels seen at presentation, yielding a total of 8 patients with down-regulation of MHC class II proteins on flow cytometry (Fig. S5A in the Supplementary Appendix).

To further extend these findings, we used immunohistochemical analysis to identify HLA-DR–positive myeloblasts in archived formalin-fixed, paraffin-embedded core samples of bone marrow from patients who were treated at our institution. Of 18 patients who had HLA-DR–positive blasts, 9 had markedly decreased expression of HLA-DR at relapse after transplantation (Fig. S7 in the Supplementary Appendix). Therefore, when these 9 patients were combined with the 8 patients with decreased expression of MHC class II proteins on flow cytometry, a total of 17 out of 34 evaluated patients with a post-transplantation relapse had evidence of down-regulation of MHC class II on either flow cytometry or immunohistochemical analysis (Fig. S8 in the Supplementary Appendix). There was no correlation between the down-regulation of MHC class II and donor

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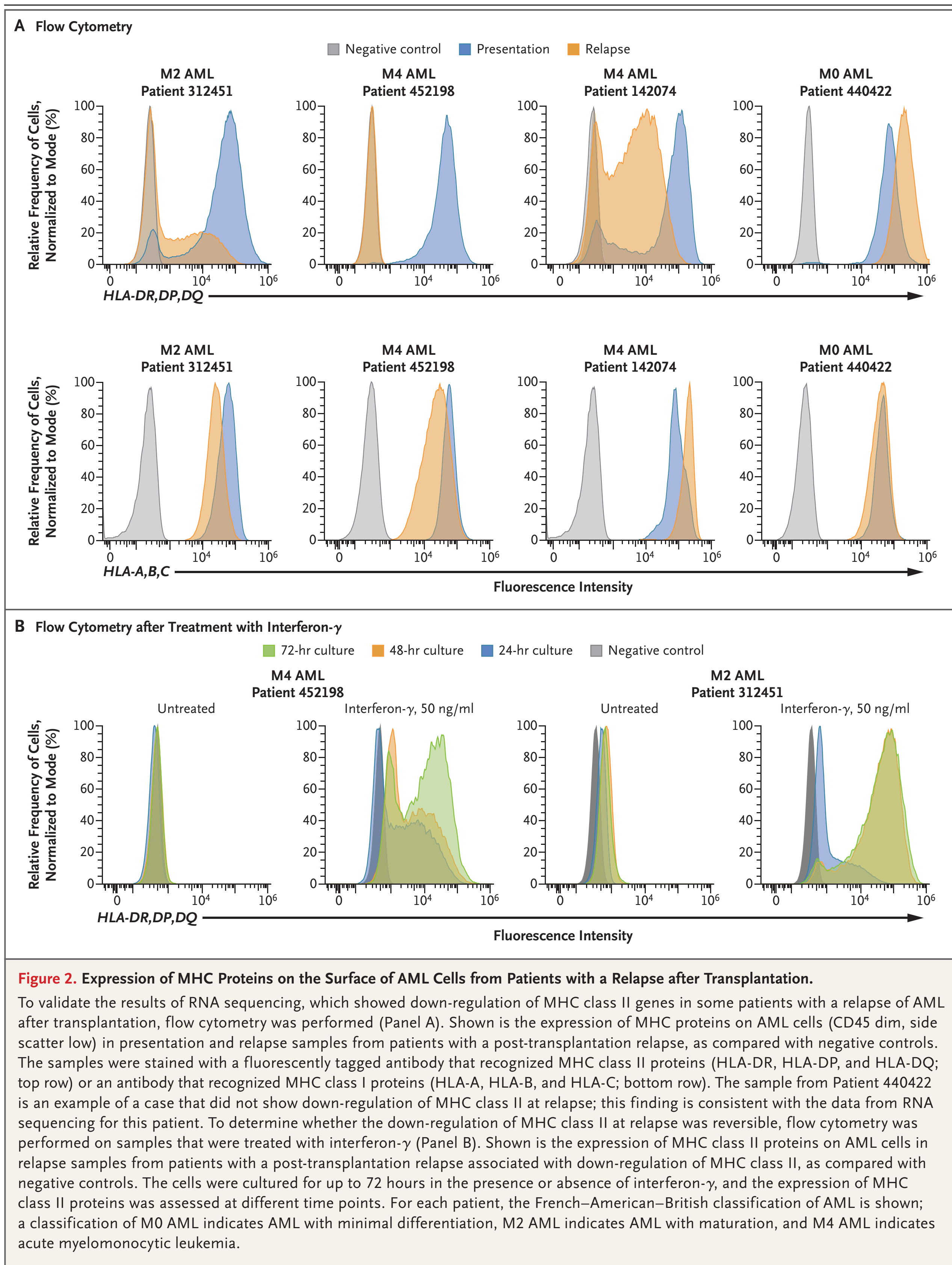

Figure 2. Expression of MHC Proteins on the Surface of AML Cells from Patients with a Relapse after Transplantation.

To validate the results of RNA sequencing, which showed down-regulation of MHC class II genes in some patients with a relapse of AML after transplantation, flow cytometry was performed (Panel A). Shown is the expression of MHC proteins on AML cells (CD45 dim, side scatter low) in presentation and relapse samples from patients with a post-transplantation relapse, as compared with negative controls. The samples were stained with a fluorescently tagged antibody that recognized MHC class II proteins (HLA-DR, HLA-DP, and HLA-DQ; top row) or an antibody that recognized MHC class I proteins (HLA-A, HLA-B, and HLA-C; bottom row). The sample from Patient 440422 is an example of a case that did not show down-regulation of MHC class II at relapse; this finding is consistent with the data from RNA sequencing for this patient. To determine whether the down-regulation of MHC class II at relapse was reversible, flow cytometry was performed on samples that were treated with interferon-γ (Panel B). Shown is the expression of MHC class II proteins on AML cells in relapse samples from patients with a post-transplantation relapse associated with down-regulation of MHC class II, as compared with negative controls. The cells were cultured for up to 72 hours in the presence or absence of interferon-γ, and the expression of MHC class II proteins was assessed at different time points. For each patient, the French–American–British classification of AML is shown; a classification of M0 AML indicates AML with minimal differentiation, M2 AML indicates AML with maturation, and M4 AML indicates acute myelomonocytic leukemia.

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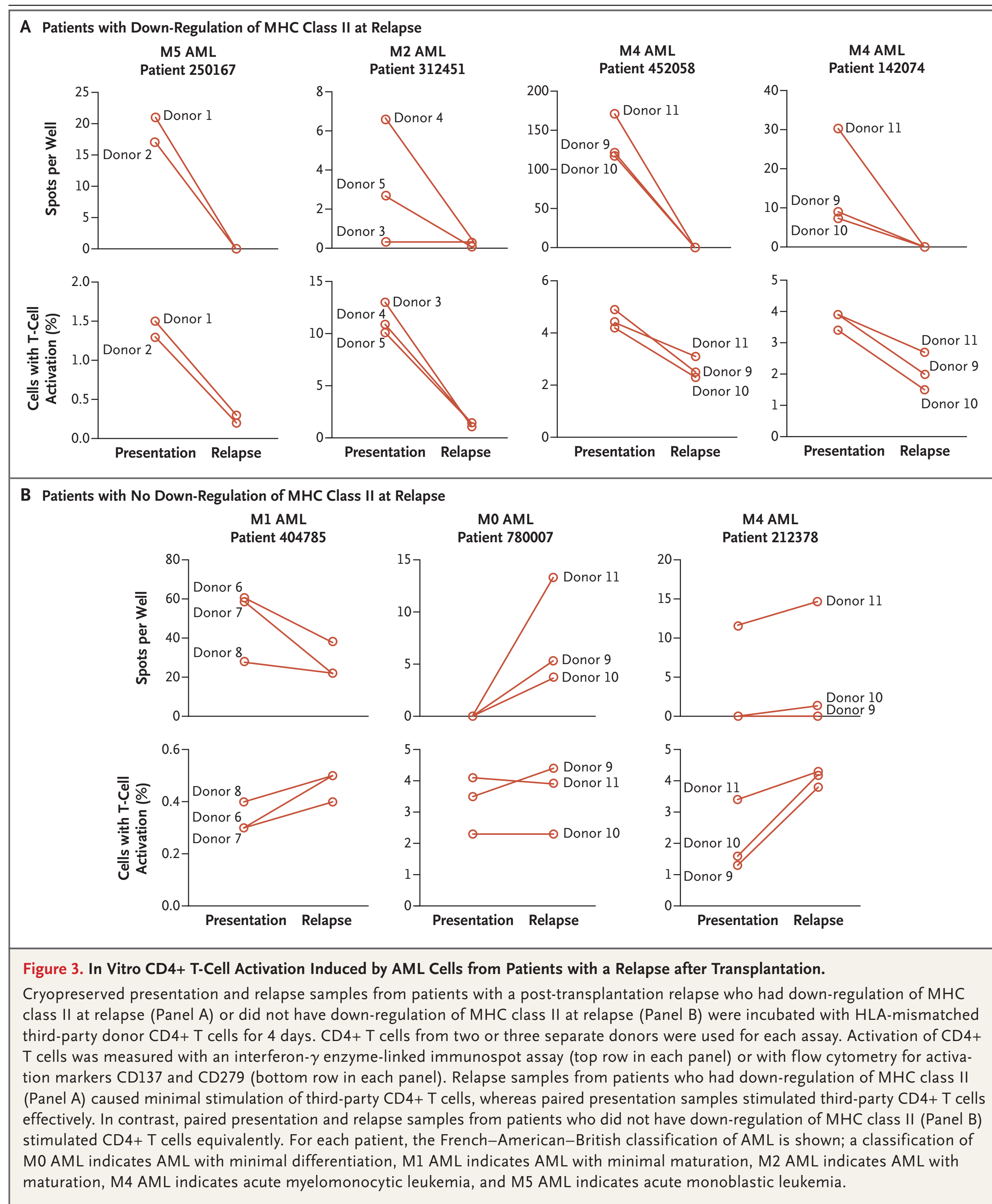

Figure 3. In Vitro CD4+ T-Cell Activation Induced by AML Cells from Patients with a Relapse after Transplantation.

Cryopreserved presentation and relapse samples from patients with a post-transplantation relapse who had down-regulation of MHC class II at relapse (Panel A) or did not have down-regulation of MHC class II at relapse (Panel B) were incubated with HLA-mismatched third-party donor CD4+ T cells for 4 days. CD4+ T cells from two or three separate donors were used for each assay. Activation of CD4+ T cells was measured with an interferon-γ enzyme-linked immunospot assay (top row in each panel) or with flow cytometry for activation markers CD137 and CD279 (bottom row in each panel). Relapse samples from patients who had down-regulation of MHC class II (Panel A) caused minimal stimulation of third-party CD4+ T cells, whereas paired presentation samples stimulated third-party CD4+ T cells effectively. In contrast, paired presentation and relapse samples from patients who did not have down-regulation of MHC class II (Panel B) stimulated CD4+ T cells equivalently. For each patient, the French–American–British classification of AML is shown; a classification of M0 AML indicates AML with minimal differentiation, M1 AML indicates AML with minimal maturation, M2 AML indicates AML with maturation, M4 AML indicates acute myelomonocytic leukemia, and M5 AML indicates acute monoblastic leukemia.

type or the use of immunosuppression at the time of relapse (Table S2 in the Supplementary Appendix).

Functional Characterization of AML Blasts

Interferon-γ has long been known to up-regulate MHC class II on a variety of cell types, including

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myeloid cells.[18,30] To determine whether the down-regulation of MHC class II genes at relapse was reversible, we used interferon-γ to treat three cryopreserved relapse samples from patients with a post-transplantation relapse associated with complete down-regulation of MHC class II proteins on flow cytometry (Patients 452198, 312451, and 142074). Culture of these cells with interferon-γ rapidly induced MHC class II protein expression on leukemic blasts, with essentially full restoration of MHC class II protein expression in nearly all AML blasts after 72 hours (Fig. 2B, and Fig. S9 in the Supplementary Appendix). The reversibility of down-regulation of MHC class II in these blasts strongly suggests that this phenomenon is mediated by an epigenetic mechanism.

To test the capacity of presentation and relapse samples of AML cells to stimulate an immune response from allogeneic T cells, we cocultured cryopreserved AML cells from seven patients with purified CD4+ T cells from HLA-mismatched third-party donors. As expected, the presentation samples (all of which had high expression of MHC class II genes) activated a subset of allogeneic CD4+ T cells, as measured by interferon-γ production and coexpression of activation markers CD137 and CD279 (Fig. 3). In contrast, the post-transplantation relapse samples that had decreased expression of MHC class II proteins had a significantly diminished capacity to stimulate third-party CD4+ T cells (Fig. 3).

Analysis of Expression of Immune-Related Genes with Single-Cell RNA Sequencing

To better characterize the expression of immune-related genes in individual AML cells, we performed single-cell RNA sequencing on bone marrow samples obtained at initial presentation and at post-transplantation relapse from Patient 452198 (a patient known in previous studies as AML31[9,31]), who had post-chemotherapy and post-transplantation relapses. Both samples were composed of more than 95% monoblasts (indicating a French–American–British classification of M5 AML).

The clonal evolution of AML at post-chemotherapy relapse and at post-transplantation relapse is shown in Figure 4A and 4B. The post-chemotherapy relapse arose from a small subclone that

Figure 4 (facing page). Clonal Evolution of AML in a Patient with a Relapse after Chemotherapy and after Transplantation.

Clonal evolution with post-chemotherapy and post-transplantation relapse was analyzed in one patient in the study (Patient 452198). Panel A shows scatter plots of somatic mutations that were found in AML cells obtained at presentation, at relapse after chemotherapy, and at relapse after transplantation according to variant allele frequency. Each data point represents the variant allele frequency of a single somatic mutation in the two indicated samples. At each time point, clusters of mutations are designated with a distinct color and shape to indicate that they represent distinct clonal populations. The mutated genes associated with each cluster are indicated in the key. Panel B shows a "fish plot" that represents the clonal evolution that can be inferred from the variant allele frequencies of somatic mutations that are shown on the scatter plots.[9] Chemotherapy began on day 0, the first relapse was detected at day 505, and the second relapse was detected at day 3269. The dominant subclone at both post-chemotherapy relapse and post-transplantation relapse was derived from a small subclone that was detected at presentation (in red), which evolved with new mutations of unknown significance after each therapy. Panels C through H show the results of single-cell RNA sequencing that was performed on cryopreserved presentation and post-transplantation relapse samples. Cells obtained at both presentation and relapse were superimposed onto a single two-dimensional plot and clustered according to their unique expression profiles with the use of t-distributed stochastic neighbor embedding (t-SNE). The axes (t-SNE1 and t-SNE2) show dimensionless values that were assigned to individual cells by the t-SNE algorithm, which places cells that have similar expression profiles close to one another. At presentation (P) and relapse (R), AML cells (AML^P and AML^R) represent the dominant cell type, and small populations of T cells (T $cell^P$ and T $cell^R$) and B cells (B $cell^P$ and B $cell^R$) can also be discerned. Panel C shows a t-SNE plot in which the cells are colored and labeled according to their inferred identity (AML^P, AML^R, B cell, or T cell); AML cells from presentation and relapse have unique expression patterns that identify them as distinct entities. In Panels D through H, the intensity of the coloring is relative to the expression of each indicated gene. In Panel D, expression of *HLA-DRA* is detected in the vast majority of AML cells at presentation but in virtually none at relapse; however, expression of *HLA-DRA* is detected in B cells at both presentation and relapse. In Panel E, expression of the housekeeping gene *GAPDH* is similar in all cell types at both presentation and relapse. In Panels F and G, expression of genes associated with T-cell exhaustion (*ICOS* and *PD1*, respectively) is detected in scattered T cells at presentation and is not increased at relapse. In Panel H, expression of the gene encoding the T-cell activation marker granzyme A (*GZMA*) is strongly detected in a subset of T cells at both presentation and relapse.

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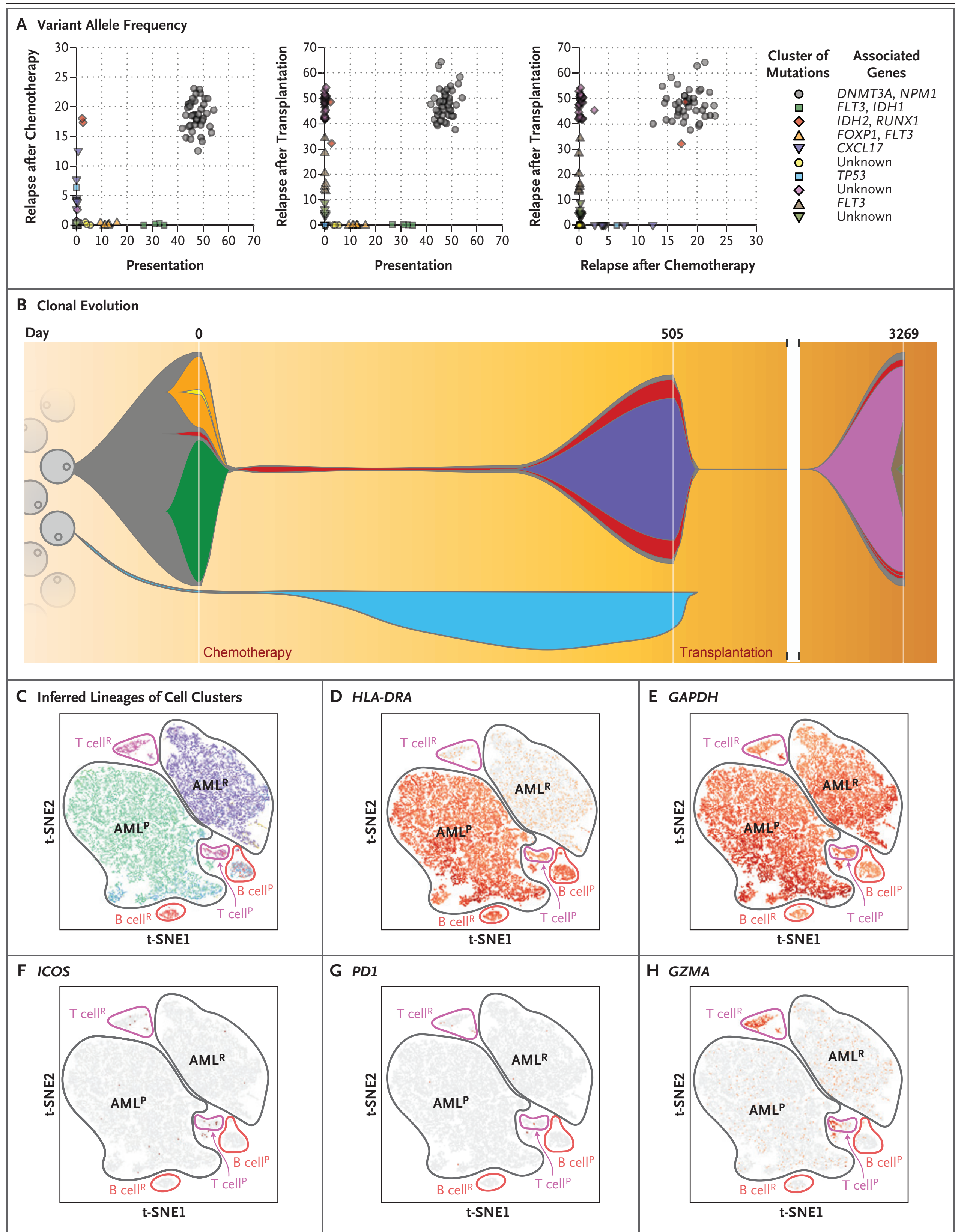

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was detected at presentation. This same subclone, which contained mutations in *IDH2* and *RUNX1*, evolved further into the post-transplantation relapse, but no new AML-specific driver mutations were present at that time and no structural variants were identified on whole-genome sequencing (Fig. 4A and 4B, and Fig. S2 and Table S4 in the Supplementary Appendix).

In Figure 4C through 4G, data from single-cell RNA sequencing are shown, with all the cells from the presentation and post-transplantation relapse bone marrow samples plotted together with t-distributed stochastic neighbor embedding (t-SNE), a graph layout algorithm that places cells with similar expression profiles near one another.[32] In this schema, there are two large cell clusters — each with a distinct expression profile — that correspond to AML cells from the presentation and post-transplantation relapse samples (Fig. 4C); small clusters with expression profiles consistent with B cells and T cells were also detected in both samples. Expression of *HLA-DRA* (and the other MHC class II genes; data not shown) was high in most of the AML cells at presentation but was virtually undetectable in all the cells at relapse (Fig. 4D), a finding consistent with the results of bulk RNA sequencing. At presentation, we did not detect a distinct cluster of *HLA-DRA*^{low} cells, which could have represented a preexisting subclone with low MHC class II gene expression.

Given the clinical interest in the use of checkpoint inhibitors to restore graft-versus-leukemia activity after transplantation,[30] we also looked for evidence of T-cell exhaustion after relapse in this patient. There was no detectable expression of *ICOS* or *PD1* in the T-cell population at relapse (Fig. 4F and 4G), even though expression of these genes was detected in a subset of T cells in normal bone marrow (Fig. S10 in the Supplementary Appendix). Expression of the gene encoding the T-cell activation marker granzyme A (*GZMA*) was detected in many T cells at presentation and at relapse (Fig. 4H); the genes encoding activation markers granzyme B and CCL5 were also expressed in those T cells (data not shown).

This combination of findings suggests that, in this patient, relapse may have been driven by rare AML cells that randomly developed a loss of MHC class II gene expression by means of an epigenetic mechanism. These cells were strongly selected for and contributed to relapse because they escaped the immune surveillance exerted by the graft-versus-leukemia effect.

DISCUSSION

Since the graft-versus-leukemia effect contributes to the therapeutic benefit of allogeneic hematopoietic stem-cell transplantation in patients with AML, we speculated that relapse of AML after transplantation might be driven by genetic changes that influence immune function. In this small sample, mutations in known immune-regulatory genes were uncommon in post-transplantation relapses, a finding consistent with the idea that such changes are not a common cause of relapse.

To determine whether post-transplantation relapse was associated with recurrent epigenetic changes, we performed an analysis of all genes with detectable RNA expression in paired samples obtained at initial presentation and at post-transplantation relapse, and we found that a large proportion of the 221 differentially expressed genes were classified as having a known or likely role in immune function. Down-regulated genes included the classical MHC class II genes, a result that confirmed findings described by Dermime et al. more than 20 years ago in three of six patients who had a relapse of chronic myeloid leukemia or AML after transplantation.[17] Since a decrease in antigen presentation could plausibly contribute to escape from the graft-versus-leukemia effect, we focused on measuring MHC class II expression in additional patients (even though it is currently unclear whether a change in this expression contributes causally to relapse). Using flow cytometry and immunohistochemical analysis, we detected decreased expression of MHC class II proteins in post-transplantation relapse samples from a total of 17 of 34 patients. The four tested post-transplantation relapse samples with decreased expression of MHC class II did not stimulate third-party CD4+ T cells in vitro. Further research would be needed to determine whether this phenomenon is sufficient to mediate AML relapse in vivo.

The reversibility of down-regulation of MHC class II by interferon-γ suggests that this event is epigenetic in nature. Single-cell RNA sequencing in one patient (Patient 452198) revealed high expression of MHC class II genes in the vast majority of AML cells at presentation. This find-

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ing suggests that immunologically resistant AML cells were rare or absent at presentation; furthermore, there was no evidence of a subclone with low MHC class II gene expression. Selection of these cells presumably occurred after the transplanted immune cells exerted selective pressure against AML cells that could be recognized immunologically. This process has been described as immunoediting in solid tumors, in which tumor clones evolve in response to immune-mediated selective pressure and ultimately escape, leading to relapse.[33] Although the use of immunosuppression after transplantation could potentially contribute to down-regulation of MHC class II, we did not detect a significant correlation between loss of MHC class II expression and use of graft-versus-host disease prophylaxis at the time of relapse (Table S2 in the Supplementary Appendix).

In conclusion, this study showed that AML cells that escaped the immune surveillance provided by allogeneic T cells after allogeneic hematopoietic stem-cell transplantation frequently had dysregulation of a number of pathways that regulate immune function. These changes appeared to be epigenetic in nature in at least some cases, which suggests that therapeutic strategies to resensitize AML cells to the graft-versus-leukemia effect may be feasible.

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APPENDIX

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