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Targeting Activation-Induced Cytidine Deaminase Overcome Tumor Evasion of Immunotherapy by CTLs

Jin-Qing Liu,*1 Pramod S. Joshi,*1 Chuansong Wang,† Hani Y. El-Omrani,* Yi Xiao,* Xiuping Liu,† John P. Hagan,† Chang-Gong Liu,† Lai-Chu Wu,‡ and Xue-Feng Bai*

Activation-induced cytidine deaminase (AID) is an enzyme essential for the generation of Ab diversity in B cells and is considered to be a general gene mutator. In addition, AID expression was also implicated in the pathogenesis of human B cell malignancies and associated with poor prognosis. In this study, we report that small interfering RNA silencing of AID in plasmacytoma dramatically increased its susceptibility to immunotherapy by CTLs. AID silencing did not decrease the mutation frequencies of tumor Ag gene P1A. Gene-array analysis showed dramatically altered expression of a number of genes in AID-silenced plasmacytoma cells, and upregulation of CD200 was shown to be in favor of tumor eradication by CTLs. Taken together, we demonstrate a novel function of AID in tumor evasion of CTL therapy and that targeting AID should be beneficial in the immunotherapy of AID-positive tumors. The Journal of Immunology, 2010, 184: 000–000.

A ctivation-induced cytidine deaminase (AID) has been identified to be an essential enzyme regulating class-switch recombination, somatic hypermutation, and gene conversion of the Ig gene in B cells (1). Ectopic expression of AID induces hypermutation in non-B cells, such as fibroblasts (2, 3), hybridomas (4, 5), and T cells (6), suggesting that AID can also target genes other than Ig. Indeed, AID transgenic mice demonstrate mutation accumulation in genes encoding TCRs, c-myc, and P53, leading to tumor development in a variety of tissues (6, 7). AID also controls c-myc/IgH translocation in B cell malignancies (8, 9) and has recently been shown to promote B lymphoid blast crisis and drug resistance in chronic myeloid leukemia (10). AID expression has been implicated in human B lineage leukemia and lymphomas, such as chronic lymphocytic leukemia (CLL) (11–13), non-Hodgkin’s lymphoma (14, 15), mantle cell lymphoma (16), follicular lymphoma (17), and Burkitt’s lymphoma (18). The detrimental role of AID has been implicated in CLL (19), and high expression of AID is a distinctive feature of poor prognosis in CLL (20). Because of the detrimental role of AID in malignant B cells and its function as a gene mutator (21, 22), targeting AID should provide beneficial effects in immunotherapy of AID-positive B cell malignancies.

Cancer Ag-specific CTLs are the major effectors against cancer cells. However, large established tumors are usually not fully controlled by CTLs because of the genetic instability of cancer cells, which often results in the selection of antigenic variants by CTLs (23–25). Using transgenic T cells specific for a natural tumor rejection Ag P1 A (P1CTL) to treat mice with large established plasmacytoma J558 tumors, we found that P1CTL selected high numbers of mutations in the P1A antigenic epitope in the plasmacytoma J558 (26, 27). Because J558 cells constitutively express the gene mutator AID, we hypothesized that targeting AID might prevent antigenic drift during P1CTL therapy of established J558 tumors. In this study, we report that small interfering RNA (siRNA) silencing of AID in plasmacytoma dramatically increased its susceptibility to immunotherapy by CTLs. Surprisingly, AID silencing did not result in reduced mutation frequencies in P1A epitope. Gene-array analysis showed alteration in expression of a number of genes, which correlated with tumor eradication. Our data suggest a critical role for AID in mediating immune evasion during T cell therapy of cancer and that targeting AID should be a useful approach for immunotherapy of AID-positive tumors.

Materials and Methods

Experimental animals and cell lines
Transgenic mice expressing a TCR specific for the tumor rejection Ag H-2Ld. P1A35-43 complex have been described previously (28). TCR transgenic mice were backcrossed with BALB/c mice for at least 10 generations before they were used for this study. BALB/c mice with a targeted mutation of the RAG-2 gene were purchased from Taconic Farms (Germantown, NY). The plasmacytoma J558 (BALB/c, H-2Ld), mastocytoma P815 (DBA/2, H-2d), and fibrosarcoma Meth A cells have been described previously (29). All cell lines were cultured in RPMI 1640 medium containing 5% FCS and 100 μg/ml penicillin and streptomycin.

Generation of AID-silenced J558 cell lines by RNA interference
Apal and EcoRI restriction sites were added to the AID targeting sequence (5′-ATTGTGGTTTCTACGCTACA-3′) at 5′- and 3′-ends. We then ligated the DNA nucleotide sequence into the Apal and EcoRI sites of pSilencer1.0 vector (U6 siRNA Expression Vector; Ambion, Austin, TX) and used it to cotransfect with a selection vector containing the Neo gene into J558 tumor cells. For control, we used empty targeting vectors and
Neo vector cotransfected J558 cells. J558 cells transfected with a negative control vector (pSilencer 4.1-CMV-neo, expressing a hairpin siRNA with no known homology to any sequences in human and mouse genome; Ambion), termed J558Sictrl, were also generated. The transfected J558 cells were selected by G418 (0.75 mg/ml) and Neo-resistant J558 clones were obtained. To verify the disruption of AID gene expression, we used RT-PCR and quantitative RT-PCR to detect AID mRNA expression and used Western blot analysis to measure AID protein expression in each selected cell clone.

**Real-time PCR**

Quantitative real-time PCR was performed using an ABI 7900 HT sequence system (Applied Biosystems, Foster City, CA) with the Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA) in accordance with the manufacturer’s instructions. PCR was done using previously determined conditions (26). The following primers were used for amplifying specific genes: mAID, 5′-TACCTTCTGTAAGACGCCGAAGGC-3′ (forward) and 5′-TTCCATG- TAGGCTTTCCAGGCTT-3′ (reverse); CD20, 5′-GAGCAGACGCTCA - GTGAGAAG-3′ (forward) and 5′-TTCGATGCACTCACATACG-3′ (reverse); Rob1, 5′-AAATXGTGCTCAGAAGCTG-3′ (forward) and 5′-GC - TGGTGAGGATTTCCAGT-3′ (reverse); AT motif binding factor 1(Atbf1), 5′-CTGGCGTGGAAGACGTCATATT-3′ (forward) and 5′-CCAAAAGTTGGA- GTTCCCCATCT-3′ (reverse); and Myb, 5′-TGATCGGAGGACCTAACAG-3′ (forward) and 5′-ATTCCATTGAGGAGACCTAC-3′ (reverse). The hypoxanthine phosphoribosyltransferase (HPRT) gene was simultaneously amplified as endogenous control. The primers were 5′-AGCTTAAGATGACGAATGGGAC-CAAATG-3′ (forward) and 5′-TTACTACGCGAGATGCCACA-3′ (reverse). The sample was assayed in triplicate, and the experiments were repeated twice. The relative amount of mRNA was calculated by plotting the Ct (cycle number), and the average relative expression for each group was determined using the comparative method (2−ΔΔCt).

**Western blot analysis**

Total protein lysates were prepared from AID-silenced J558 cells and controls using M-PER mammalian protein extraction reagent (Pierce, Rockford, IL). Protein extracts were resolved by 4–20% SDS-PAGE and transferred onto nitrocellulose membranes (Millipore) using M-PER mammalian protein extraction reagent (Pierce, Rockford, IL). Total protein lysates were prepared from AID-silenced J558 cells and controls. Western blot analysis was used to measure AID protein expression in each selected cell clone.

**FIGURE 1.** Plasmacytoma J558 cells constitutively express mutator AID and evade P1CTL therapy via Ag drift. A, Rejection and recurrence of J558 tumors after P1CTL therapy. A total of 5 × 10⁶ of J558 cells were injected into the flank of each male BALB/c mouse s.c. When tumors grew into a size of 0.6–0.8 cm in diameter, mice were injected with purified P1CTL cells i.v. at a dose of 5 × 10⁶/mouse. Eleven mice were used in this experiment. B, Summary of mutation sites (indicated as bold nucleotides) in P1A gene (nucleotide numbers are relative to the translation starting site of P1A mRNA). Data are based on the analysis of 12 P1CTL-escape J558 clones. C, Each of the mutations indicated in B resulted in a single amino acid change in the P1CTL-recognized epitope (indicated in bold). D, RT-PCR (Cr = 30) was performed using cDNAs from B cell lymphoma A2, plasmacytoma J558, fibrosarcoma Meth A, and mastocytoma P815 cells. The primers used for AID amplification were 5′-GTCAAGAAGATCGCTCAGGAGG-3′ (forward) and 5′-ACGGTTGACATTGCGAGTTGCTGT-3′ (reverse). The primers used for GAPDH amplification were 5′-ATGGTGAGGTTCGTTGAGTGAAC-3′ (forward) and 5′-GAGCACAGCTCA-GCCAGAAAT-3′ (reverse). Ten nanograms of genomic DNA sample was amplified for a standard 40-cycle TaqMan PCR amplification (Applied Biosystems) according to the manufacturer’s instructions. Genomic DNA samples from both control and AID-silenced cell lines were amplified. DNA from a clonal J558 cell line (J558T2S) that specifically bears N389T > C mutation (26) was used as positive control. Moreover, by mixing serial numbers of the mutated J558T2S cells with fixed numbers (1 × 10⁶) of wild-type J558 cells, a standard curve for the calculation of mutation frequency was generated based on the linear relationship between log number of J558T2S cells (mutated) and amplification Ct difference (ΔCt = Ctmutated − Ctwt). On the basis of the actual ΔCt value of each cell line, we calculated the mutation frequency using the following formula: mutation frequency (f) = 10−ΔCt/6.

**Quantification of P1A gene mutation frequencies**

We used Real-Time TaqMan PCR to detect P1A gene mutation at N389 relative to the P1A mRNA. We have frequently detected a T > C mutation in the P1A gene at this position in J558 cells that evaded P1CTL therapy. Two TaqMan probes were designed to simultaneously detect mutated (C) and wild-type (T) alleles at this position. The probe sequences used were mP1A-F389 (FAM) 5′-TGCCCTATCTGAGGCGG-3′ (for the detection of N389-C) and mP1A-F389 (VIC) 5′-TCTGCTATCTGAGGCGG-3′ (for the detection of N389-T). The primers used were 5′-ACGGTTGACATTGCGAGTTGCTGT-3′ (forward) and 5′-GAGCACAGCTCA-GCCAAAT-3′ (reverse). We used Real-Time TaqMan PCR to detect P1A gene mutation at N389 relative to the P1A mRNA. We have frequently detected a T > C mutation in the P1A gene at this position in J558 cells that evaded P1CTL therapy. Two TaqMan probes were designed to simultaneously detect mutated (C) and wild-type (T) alleles at this position. The probe sequences used were mP1A-F389 (FAM) 5′-TGCCCTATCTGAGGCGG-3′ (for the detection of N389-C) and mP1A-F389 (VIC) 5′-TCTGCTATCTGAGGCGG-3′ (for the detection of N389-T). The primers used were 5′-ACGGTTGACATTGCGAGTTGCTGT-3′ (forward) and 5′-GAGCACAGCTCA-GCCAAAT-3′ (reverse). We used Real-Time TaqMan PCR to detect P1A gene mutation at N389 relative to the P1A mRNA. We have frequently detected a T > C mutation in the P1A gene at this position in J558 cells that evaded P1CTL therapy. Two TaqMan probes were designed to simultaneously detect mutated (C) and wild-type (T) alleles at this position. The probe sequences used were mP1A-F389 (FAM) 5′-TGCCCTATCTGAGGCGG-3′ (for the detection of N389-C) and mP1A-F389 (VIC) 5′-TCTGCTATCTGAGGCGG-3′ (for the detection of N389-T). The primers used were 5′-ACGGTTGACATTGCGAGTTGCTGT-3′ (forward) and 5′-GAGCACAGCTCA-GCCAAAT-3′ (reverse). We used Real-Time TaqMan PCR to detect P1A gene mutation at N389 relative to the P1A mRNA. We have frequently detected a T > C mutation in the P1A gene at this position in J558 cells that evaded P1CTL therapy. Two TaqMan probes were designed to simultaneously detect mutated (C) and wild-type (T) alleles at this position. The probe sequences used were mP1A-F389 (FAM) 5′-TGCCCTATCTGAGGCGG-3′ (for the detection of N389-C) and mP1A-F389 (VIC) 5′-TCTGCTATCTGAGGCGG-3′ (for the detection of N389-T). The primers used were 5′-ACGGTTGACATTGCGAGTTGCTGT-3′ (forward) and 5′-GAGCACAGCTCA-GCCAAAT-3′ (reverse). We used Real-Time TaqMan PCR to detect P1A gene mutation at N389 relative to the P1A mRNA. We have frequently detected a T > C mutation in the P1A gene at this position in J558 cells that evaded P1CTL therapy. Two TaqMan probes were designed to simultaneously detect mutated (C) and wild-type (T) alleles at this position. The probe sequences used were mP1A-F389 (FAM) 5′-TGCCCTATCTGAGGCGG-3′ (for the detection of N389-C) and mP1A-F389 (VIC) 5′-TCTGCTATCTGAGGCGG-3′ (for the detection of N389-T). The primers used were 5′-ACGGTTGACATTGCGAGTTGCTGT-3′ (forward) and 5′-GAGCACAGCTCA-GCCAAAT-3′ (reverse). We used Real-Time TaqMan PCR to detect P1A gene mutation at N389 relative to the P1A mRNA. We have frequently detected a T > C mutation in the P1A gene at this position in J558 cells that evaded P1CTL therapy. Two TaqMan probes were designed to simultaneously detect mutated (C) and wild-type (T) alleles at this position. The probe sequences used were mP1A-F389 (FAM) 5′-TGCCCTATCTGAGGCGG-3′ (for the detection of N389-C) and mP1A-F389 (VIC) 5′-TCTGCTATCTGAGGCGG-3′ (for the detection of N389-T). The primers used were 5′-ACGGTTGACATTGCGAGTTGCTGT-3′ (forward) and 5′-GAGCACAGCTCA-GCCAAAT-3′ (reverse).
Tumorigenesis and P1CTL adoptive transfer therapy of mice with established tumors

For tumor establishment in vivo, 5 × 10^6 of J558 cells or 1 × 10^6 of P815 cells or their variant cells were injected into each mouse s.c. Tumor volumes were measured along three orthogonal axes (a, b, and c) every 3 d and calculated as a × b × c/2 (31). For CTL therapy of mice with established tumors, pools of spleen and lymph node cells from P1CTL-transgenic mice were incubated with a mixture of mAbs (anti-CD4 mAb GK1.5, anti-CD8 mAb 2.4G2, and anti-CD11c mAb N418). After removal of unbound mAbs, cells were incubated with anti-Ig-coated magnetic beads (Dynal Biotech, Carlsbad, CA). The Ab-coated cells were removed by a magnet. The unbound cells consisted of >90% CD8+ T cells, with no detectable CD4+ T cells. The purified CD8+ T cells (5 × 10^6/mouse) were injected i.v. into mice bearing established tumors.

Mutation analysis

PCR-based Topo cloning and sequencing were performed on P1A, GAPDH, and IgL chain V segment (32, 33). The primers used were as follows: P1A, 5'-GCTAGCTGCGGACTACTTATCT-3' (forward) and 5'-TGCAACAGTCATGCTAAAGTGAG-3' (reverse); GAPDH, 5'-ATGGTGAAGGTC-3' (forward) and 5'-GACTGGTGATTTGGC-3' (reverse). PCR products were cloned into the ApaI and EcoRI sites of pSilencer1.0 vector (U6 siRNA Expression Vector; Ambion) and used to cotransfect with a selection vector containing the Neo gene into J558 tumor cells. As control, we used empty targeting vectors and Neo vector cotransfected J558 cells. The transfected J558 cells were then selected by G418 (0.75 mg/ml), and Neo-resistant J558 clones were obtained.

Statistical analysis

Tumor rejection rates in different groups of mice were compared using Fisher’s exact test. Student t test was used to compare size differences between two groups. For comparison of mice survival, the Kaplan-Meier survival analysis and log-rank test were used (version 10.0; SPSS, Chicago, IL). A value of p < 0.05 was considered significant.

Results

Plasmacytoma J558 tumors constitutively express gene mutator AID and evade CTL therapy via Ag drift

Using transgenic T cells specific for a natural tumor rejection Ag P1A (P1CTL) to treat mice with large established plasmacytoma J558 tumors, we found that P1CTL therapy was highly effective initially, as reflected in Fig. 1A. About 1 wk after P1CTL injection, we started to observe dramatic tumor volume reduction, and the tumor rejection process typically lasted ∼4 wk. However, after 4 wk, ∼100% of tumors resumed growing. In our previous studies (26, 27), we have found that the failure of P1CTL therapy for J558 tumors was due to various point mutations in the P1A antigenic epitope, termed Ag drift. We have performed extensive analysis of 12 P1CTL-escape J558 tumors, and overall, we detected eight mutations in the P1A epitope. The mutation locations relative to the translation starting site of P1A mRNA were indicated in Fig. 1B. The single nucleotide substitutions resulted in seven amino acid changes at 4, 5, 6, 7, 8, and 9 positions of P1A 35-43 epitope, respectively (Fig. 1C). Despite the multiple mutations detected, a few mutations were dominant over the others. P6R, which is due to the T→C mutation at nt 389 of P1A mRNA, had the highest frequency. Among the 12 J558 variants, nine tumors had this type of mutation. These mutations severely diminished T cell recognition of the tumor Ag by a variety of mechanisms, including modulation of MHC:peptide interaction and TCR binding to MHC:peptide complexes, which were previously shown to be sufficient to cause tumor evasion of destruction by P1CTL (26, 27). This mechanism (Ag drift) of
immune evasion, however, rarely occurred in mastocytoma P815 and fibrosarcoma Meth A tumors, which shared the same tumor Ag P1A (26, 27). In searching for the mechanism of immune evasion of cancer cells, we found that the J558 cells, but not P815 and Meth A cells, constitutively expressed the gene mutator AID (Fig. 1D). J558 cells express Ig L chain (32, 33), and sequencing analysis of the V segment of L chain revealed a high mutation frequency (∼8 × 10⁻⁵/hp), which typically occurred in G–C pairs (Fig. 1E). Thus, AID expressed in J558 cells was functional.

**AID-silenced J558 tumors exhibit increased susceptibility to CTL therapy**

Because AID is a gene mutator that targets a variety of genes, we tested whether AID mediated P1A Ag drift during CTL therapy of J558 tumors. We first generated J558 cells with stably silenced AID gene expression. As shown in Fig. 2A, control vector transfected J558 cells (C4 and C5) constitutively expressed the AID gene, whereas expression of the AID gene was almost undetectable in targeting vector-transfected cells. The targeting effect was AID specific, as the expression of tumor rejection Ag P1A gene was not affected by the targeting vector transfected in these cells. The J558 cells with disrupted AID gene expression were further tested by quantitative PCR (Fig. 2B). The reduction of AID gene expression in silenced J558 cells ranged from 17.1- to 121.8-fold if compared with clone C4. Western blot analysis verified that AID expression was dramatically reduced or undetectable in silenced cells at the protein level (Fig. 2C). AID-silenced cell lines expressed similar levels of H-2Ld as compared with control cell lines C4 and C5 (Fig. 2D). Moreover, these cell lines can be efficiently recognized by P1A-specific CTL in vitro (Fig. 2E). AID disruption did not significantly alter the growth and survival of J558 cells, because similar proliferation was detected among AID-silenced J558 cells and control cells (Fig. 2F). Tumorigenicity tests suggested that all AID-disrupted J558 cells grew into large tumors in RAG-2⁻/⁻ BALB/c mice (Fig. 3A). In addition, cells from ex vivo tumors retained the AID-disrupted status (Fig. 3B), suggesting that the AID silencing was maintained in vivo in established tumors. To test whether established tumors formed by AID-silenced J558 cells can be eliminated by P1A-specific CTLs in vivo, we injected AID-silenced J558 cells or control J558 cells (AID⁺) into RAG-2⁻/⁻ BALB/c mice. Once tumors grew to ∼0.6–0.8 cm in diameter, we injected 5 million purified P1CTL cells into each recipient mouse. As shown by a representative experiment in Fig. 3C, all tumor-bearing mice responded to T cell therapy, as reflected by dramatic tumor volume reduction, which typically occurs 5–7 d after T cell transfer. By 2 wk after P1CTL therapy, tumors in all groups were reduced to minimal size. By 4 wk after T cell therapy, five of five tumors resumed growth in mice with C4 or C5 tumors. In contrast, we observed continuous tumor volume reduction in AID-silenced J558 tumors (AID 2.17 and AID 2.27), and as a result, mice bearing AID-silenced tumors had significantly prolonged survival (Fig. 3D). As summarized in Fig. 3E, 25–90% of AID-silenced J558 tumors were completely rejected after P1CTL therapy, whereas control tumors were rarely rejected (0% for parental J558 and C5 tumors; 8% for C4 tumors). The complete rejection of AID-silenced tumors by CTL was not due to nonspecific expression of siRNA in tumor cells, because tumors expressing a nonspecific siRNA (J558Sictrl) were not rejected (Fig. 3C–E). Thus, AID silencing facilitated better tumor rejection by P1CTL.

**FIGURE 3.** Downregulation of AID expression in plasmacytoma J558 cells facilitates tumor rejection by P1CTL. A. Tumor growth kinetics in RAG-2⁻/⁻ BALB/c mice. A total of 5 × 10⁶ AID-silenced J558 cells or controls were injected into the flank of each RAG-2⁻/⁻ BALB/c mouse s.c. Three to five mice per group were used, and data were pooled from two experiments. B. RT-PCR was used to measure expression of AID gene from ex vivo tumors. A total of 5 × 10⁶ of AID-silenced J558 cells or controls were injected into RAG-2⁻/⁻ BALB/c mice, and established tumors were analyzed for AID expression. C. Rejection and recurrence of AID-silenced J558 tumors and controls after P1CTL therapy. A total of 5 × 10⁶ AID-silenced J558 cells (2.17 or 2.27) or controls (C4, C5, and J558Sictrl) were injected into the flank of each RAG-2⁻/⁻ BALB/c mouse s.c. When tumors grew into a size of ∼0.6–0.8 cm in diameter, mice were injected with purified P1CTL cells i.v. at a dose of 5 × 10⁶/mouse. Five mice per group were used in this experiment. D. Survival of different groups of mice after P1CTL therapy. Data from the same groups of mice from C were shown. 2.17 and 2.27 versus J558Sictrl; p = 0.0031. E. Summary of tumor rejection after P1CTL therapy. All mice were treated as described in C. Data were pooled from three experiments. J558Sictrl, J558 cells transfected with a vector expressing a negative control siRNA.
AID silencing does not reduce mutation frequency of P1A Ag in J558 cells

To determine whether a lack of tumor recurrence is due to lower tumor mutation frequency in the P1A antigenic epitope in AID-silenced cells, we developed an in vitro assay to measure P6R (T > C mutation at nt 389 relative to P1A mRNA) mutation frequencies in AID-silenced J558 cells and controls. P6R mutation has been shown (26, 27) to be of the highest incidence among all the mutations detected in P1CTL-escape J558 cells. A TaqMan real-time PCR allowed the detection of the wild-type and mutated P1A gene simultaneously (Fig. 4A). By mixing variable numbers of P6R-mutated cells with wild-type J558 cells, we were able to generate a standard curve and used it to calculate P6R mutation frequencies for different cells (Fig. 4B). Surprisingly, P6R mutation frequencies were higher or unchanged in all the AID-silenced J558 clones compared with their relative controls, ranging from 1.9- to 21.9-fold (Fig. 4C). To determine whether the increased mutation frequency of P1A was the result of increased spontaneous mutation of genes in AID-silenced J558 cells, we measured the mutation frequencies of J558 cells at the HPRT locus, we used high concentrations of 6-TG to treat AID-silenced J558 cells and their relative controls. TG treatment would permit the selection of “nonleaky” mutations at the HPRT locus (34–36). These mutations, which are permissive of sustained cellular proliferation, could presumably have arisen from base pair substitutions (point mutation), frame shifts, and small deletions (34). As demonstrated in Fig. 4D, more TG-resistant clones arose from AID-silenced J558 cells than controls. Sequencing analysis of HPRT cDNA suggested that all (eight of eight) 6-TG-resistant AID-2.17 cells (5 μg/ml 6-TG treated) contained at least one point mutation (Fig. 4E). Thus, AID-silencing paradoxically increased mutation frequencies at both P1A and HPRT loci; therefore, reduction of Ag mutation frequency in AID-silenced J558 cells is not responsible for their enhanced rejection by P1CTL. In line with these findings, we found that AID-silenced J558 tumors that escaped P1CTL therapy also mutated their P1A Ag (Fig. 5). P1A Ag from four recurrent C4 tumors (AID positive) and four recurrent AID-silenced tumors were cloned and sequenced, and we detected three previously reported mutations (26, 27), as demonstrated in Fig. 5A, P6R mutation was found in seven of eight recurrent tumors and dominated in both AID-positive and AID-silenced tumors (Fig. 5B).

Altered gene expression profiling in AID-silenced J558 cells

To further determine the potential mechanisms by which AID elimination facilitate better J558 tumor rejection by P1CTL, we performed cDNA microarray analysis to compare AID-silenced J558 cells and their relative controls (GEO accession number GSE19110). We have observed significantly altered gene expression profiling between AID-silenced J558 cells and controls (Fig. 6). By comparing results from two independent experiments (C4 versus 2.27, C5 versus 2.13) (Fig. 6A, 6B), we identified 18 genes whose expression in AID-silenced J558 cells had at least 4-fold changes (Fig. 6C).

Among the most dramatically changed genes in AID-silenced J558 cells were CD200, Robo1, and Atbf1. Quantitative RT-PCR (Fig. 7A) and RT-PCR (Fig. 7B) verified strong upregulation of...
CD200 and Robo1 and downregulation of Atbf1 genes in all AID-silenced J558 cells compared with control AID+ cells. Atbf1 is a large transcription factor (37, 38) and has been shown to negatively regulate the expression of Myb oncoprotein (39) and is considered to be a tumor suppressor gene (40). In this study, we detected a 1500-fold reduction of Atbf1 in all the AID-silenced J558 cells (Fig. 7A), consistent with downregulation of Atbf1, we also detected upregulation of Myb (Fig. 7A). CD200 is a highly conserved type 1 transmembrane glycoprotein (41). The expression of the receptor for CD200 (CD200R) is restricted to myeloid-derived APCs and certain populations of T cells (42). The major function of CD200 has been described as downregulation of the function of myeloid lineage cells (43). Direct cell surface staining and flow cytometry analysis verified that CD200 is expressed on all AID-silenced J558 cells but expressed in none or a small number of control AID+ J558 cells (Fig. 7C). To test whether upregulation of CD200 was responsible for the tumor rejection by CTLs, we injected CD200-positive 2.13 cells into RAG-2−/− BALB/c mice. When tumors were fully established, we treated tumor-bearing mice with P1CTL, followed by intratumor injection of four doses of anti-CD200 Abs or isotype-matched control Abs. As shown in Fig. 7D, injection of CD200 Ab into 2.13 tumors during CTL therapy significantly enhanced tumor recurrence. Six of 10 control Ab-treated mice rejected 2.13 tumors, whereas only 3 of 10 anti–CD200-treated mice rejected 2.13 tumors, and the size of tumors was significantly different between the two groups 33 d after T cell treatment (p < 0.01).

**Discussion**

In this study, we have made the following three observations: first, we have demonstrated that siRNA silencing of AID in plasma-cytoma dramatically increased its susceptibility to immunotherapy by CTL. Second, AID silencing did not reduce mutation frequency of tumor rejection Ag P1A but rather altered gene expression profiling in J558 cells. Third, increased expression of some genes (such as CD200) in AID-silenced J558 cells was beneficial for preventing tumor recurrence.

The role of AID in mediating tumor Ag mutation in J558 cells

Using P1CTL to treat mice with large established plasmacytoma J558 tumors, we found that P1CTL selected high numbers of mutations in the P1A antigenic epitope (Fig. 1B, 1C). In contrast to J558 cells, we have found that T cell therapy of the other two lineages of P1A+ tumors (Meth A and P815) rarely cause P1A antigenic drift (27). Importantly, J558 tumor cells constitutively express AID (Fig. 1D), and high frequencies of G/C mutations were detected in the V segment of IgG L chain in J558 cells (Fig. 1E), suggesting that the cytidine deaminase activity of AID is active. These observations suggest AID may directly target tumor Ag P1A and mediates “Ag drift” in the P1A Ag epitope. Despite these possible links, accumulating evidences exist and do not support this hypothesis. First, the Ag drift mutations detected in P1A+ tumors (Meth A and P815) rarely cause P1A antigenic drift (27). Importantly, J558 tumor cells constitutively express AID (Fig. 1D), and high frequencies of G/C mutations were detected in the V segment of IgG L chain in J558 cells (Fig. 1E), suggesting that the cytidine deaminase activity of AID is active. These observations suggest AID may directly target tumor Ag P1A and mediates “Ag drift” in the P1A Ag epitope. Despite these possible links, accumulating evidences exist and do not support this hypothesis. First, the Ag drift mutations detected in P1A+ tumors (Meth A and P815) rarely cause P1A antigenic drift (27).

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** AID-silenced J558 tumors that escaped P1CTL therapy had similar point mutations in the tumor Ag P1A. P1A cDNA from each recurrent tumor was first amplified by PCR, followed by Topo cloning. P1A cDNA clones from each recurrent tumor were then sequenced. A, Chromatograms of sequencing reactions of P1A Ag epitope-coding region. Three types previously identified mutations (P6R, PSV, and P8L) in the P1A epitope-coding region were detected in recurrent tumors. Arrows indicate the positions where mutations occurred. B, Summary of mutation distribution in recurrent AID-silenced J558 cells and controls. P6R mutation was the dominant one detected in both AID-silenced J558 cells and controls.

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** Microarray analysis reveals genes regulated by AID. The mouse Affymetrix Mouse Genechips 430-2.0 arrays were hybridized to RNA derived from control and AID-silenced J558 cells according to the manufacturer’s directions. Two different pairs of cells (C4 versus 2.27 (A) and C5 versus 2.13 (B)) were used, leading to the identification of nine upregulated genes and nine downregulated genes that were shared by two AID-silenced J558 clones (C) whose expression differed by at least 4-fold for both comparisons.
of genes and were somehow higher in AID-silenced J558 cells compared with controls, as we also detected increased mutation frequencies in HPRT gene in those cells. Finally, we found that AID-silenced tumors that escaped P1CTL therapy also had similar point mutations as those found in recurrent AID-positive tumors (Fig. 5), thus AID silencing was not preventing Ag drift in vivo.

The role of AID in regulating gene expression profiling

We have found AID silencing is associated with unique gene expression profiling (Fig. 6) in plasmacytoma cells. However, it remains unclear how AID silencing resulted in the expression of a set of new genes and downregulation of others. Because AID is a mutator and is shown to mediate mutation of tumor suppressor genes TP53 (7) and CDKN2B (21), it is possible that AID may mutate some “master genes,” which in turn can regulate expression of other genes. In this case, P53 has been shown to regulate CD200 expression in dendritic cells (44). Although TP53 gene expression is not altered in AID-silenced J558 cells, it remains to be determined whether AID-positive tumor cells accumulated more mutations in TP53 than in AID-silenced cells. Another notable observation in this study is that a tumor suppressor gene, Atbf1, which is known to repress the expression of a number of oncogenes, such as myb (39, 40), is strongly downregulated in AID-silenced J558 cells. It remains to be determined whether downregulation of atbf1 and the resulting upregulation of oncogene myb (Fig. 7A) are responsible for the altered gene expression profiling in AID-silenced J558 cells. Finally, recent evidences (45–47) suggest that AID is a genome-wide eraser of DNA methylation, and this function of AID correlates with the current observation that AID silencing alters gene expression profiling in plasmacytoma J558 cells.

P1CTL-mediated tumor rejection mechanisms in AID-silenced J558 tumors

In this study, we have demonstrated that siRNA silencing of AID in plasmacytoma dramatically increased its susceptibility to immunotherapy by CTLs. We propose that the alteration of gene expression profiling of AID-silenced cancer cells is responsible for the improved outcome of CTL therapy. We have shown that upregulation of CD200, a B7 family of molecule on AID-silenced J558 tumor cells is beneficial for tumor eradication, as blockade of CD200–CD200R interaction resulted in tumor recurrence (Fig. 7). The major function of CD200 is to interact with CD200R on tumor-associated macrophages (TAMs) to inhibit their functions. TAMs are usually of the M2 phenotype (producing IL-10) (48, 49) and known to suppress CTL effector functions and promote tumor invasion.
growth, and angiogenesis (50–52). Targeting TAMs alone has been shown to be effective therapy in tumor models (53, 54). In addition to CD200, upregulation of Robo1, a cell surface receptor, may potentially inhibit tumor angiogenesis via competitive inhibition of Robo1 on endothelial cells to interact with its ligand Slit2 (55). Thus, upregulation of CD200 and Robo1 can result in altered tumor microenvironment that not only enhances the effect factors of adaptively transferred CTL but also inhibits tumor angiogenesis. As previous studies (31, 56, 57) have revealed, powerful CTLs can kill both antigenic tumor cells and tumor stromal cells that cross-present tumor Ag. This mechanism will result in the tumor bed destruction, leading to antigenic variant cancer cells fail to survive and grow out. Enhanced IFN-γ production by CTLs can also inhibit angiogenesis in tumor bed and results in tumor rejection (58, 59). Thus, complete rejection of AID-silenced tumors by adaptively transferred CTLs is due to both tumor Ag-dependent mechanisms (CTL-mediated cognate destruction of tumor and stromal cells) and nontumor Ag-dependent mechanisms (inhibition of tumor angiogenesis/eradication of antigenic variants).

Recently, AID has been shown (10) to promote drug resistance in chronic myeloid leukemia models. Although our cytotoxicity assay did not reveal much difference in sensitivity to PI-CTL lysis among AID-silenced J558 cells and controls in vitro (Fig. 2E), we do not rule out the possibility that AID-silenced J558 cells are more susceptible to apoptotic insults in vivo. Although some of these possibilities remain to be tested, we consider that the alteration of gene expression profiling in AID-silenced tumor cells significantly changed the susceptibility of tumors to CTL therapy. Taken together, our data have revealed a novel function of AID in host evasion of T cell therapy and suggest that targeting AID may be beneficial in immunotherapy of AID-positive malignancies.

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Disclosures

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