

but are complementary to X_1 and X_2 , respectively). Fragmented cDNAs of each sample were ligated to our custom paired-end adaptors, and the 'two-base tag' of N_1N_2/X_1X_2 sequences was used to assign read data to the original sample. Given the availability of 16 independent two-base codes, a single lane of the Illumina flow cell can simultaneously run up to 16 samples. The *MAP2K1* mutations identified in the GAIIX data sets were further confirmed by genomic PCR analysis of the corresponding regions of the gene followed by Sanger sequencing.

Results

Identification of *MAP2K1(Q56P)* in *OCUM-1* cells

The scirrhous subtype of gastric carcinoma (diffusely infiltrating carcinoma or Borrmann type IV) is a highly infiltrating poorly differentiated gastric cancer with a 5 year survival rate of only 11.3% (13). Despite the discovery of germ-line mutations in *CDH1* associated with familial diffuse-type gastric cancer (14), such mutations are present at only a relatively low frequency among sporadic cases, and little is known of the driver mutations for this intractable disorder. To identify transforming genes in scirrhous gastric cancer, we constructed a cDNA expression library from a cell line (OCUM-1) established from an individual with this condition. A focus formation assay performed with 3T3 cells infected with the retroviral library resulted in the identification of a dozen transformed foci. Recovery and nucleotide sequencing of insert cDNAs from such foci revealed that one of the cDNAs was derived from human *MAP2K1* with a nucleotide substitution of A-to-C at position 642 of the cDNA (GenBank accession number, NM_002755). Sequencing of this position in OCUM-1 genomic DNA confirmed the presence of this substitution, which results in replacement of a glutamine at amino acid position 56 with proline (Supplementary Figure 1 is available at *Carcinogenesis* Online). *MAP2K1*, also known as MEK1, functions immediately upstream of MAPK in the MAPK signaling pathway.

Transforming ability of *MAP2K1(Q56P)*

The *MAP2K1(Q56P)* mutation was first detected by *in vitro* screening to identify activated components of the RAS-MAPK signaling

cascade in rat fibroblasts (15), and it was later identified in a human lung cancer cell line (16). We confirmed the transforming ability of *MAP2K1(Q56P)* by using a focus formation assay with mouse 3T3 fibroblasts (Figure 1A) and a tumorigenicity assay with nude mice (Figure 1B). Furthermore, such potential of *MAP2K1(Q56P)* was canceled by eliminating its enzymatic activity (through a replacement of lysine-97 in the adenosine triphosphate-binding pocket to a methionine) or by incubation with a selective *MAP2K1/2* inhibitor, AZD6244 (17).

Although the Q56P mutation was previously shown to confer an ~10-fold increase in the enzymatic activity of *MAP2K1* (15), little has been known of the dependence of cancer cells on *MAP2K1(Q56P)* for viability. To address this issue, we compared the effect of AZD6244 on OCUM-1 cells with that on two other cell lines of human scirrhous-type gastric cancer, KATOIII and NUGC-4. Sanger sequencing of genomic DNA from the latter two cell lines confirmed the absence of mutations that could give rise to the Q56P substitution (data not shown). Treatment with AZD6244 inhibited the proliferation of OCUM-1 cells but not that of KATOIII and NUGC-4 cells (Figure 2A), showing that *MAP2K1(Q56P)* is a suitable target for drug development. Furthermore, in OCUM-1 cells, phosphorylation of ERK1/2 was substantially decreased upon the AZD6244 treatment (Figure 2B) but such effect was less prominent in the other cell lines.

Multiplex deep sequencing of *MAP2K1* cDNAs

Given the dependence of OCUM-1 cells on *MAP2K1(Q56P)* for survival, we next searched for transforming *MAP2K1* mutations in other epithelial tumors. It should be noted, however, that epithelial tumors are frequently contaminated with inflammatory cells and fibrous or necrotic tissue (especially in the case of infiltrative scirrhous-type gastric cancer), which makes it difficult to determine the presence or absence of *MAP2K1* mutations with the use of conventional Sanger sequencing. We therefore chose a deep sequencing approach with the use of a next-generation sequencer. To facilitate simultaneous sequencing of multiple samples, we added two-base tags to the

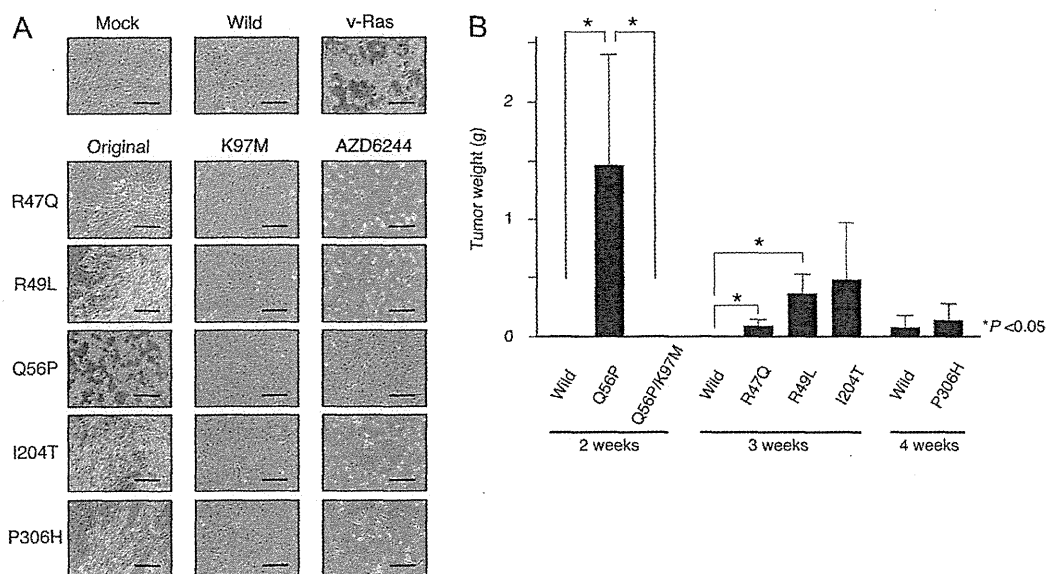


Fig. 1. Transforming potential of *MAP2K1* mutants. (A) Recombinant retroviruses encoding *MAP2K1* (Wild), the indicated *MAP2K1* mutants or v-Ras or the corresponding empty virus (Mock), were used to infect 3T3 cells, which were then cultured for 13 days and photographed. Scale bars, 400 μ m. The kinase-dead form (K97M) of each mutant was similarly analyzed. In addition to the cells expressing original mutants, the same set of cells was separately treated with 400 nM of AZD6244 for 13 days. (B) Mouse 3T3 cells expressing wild-type *MAP2K1*, Q56P or its kinase-dead form (Q56P/K97M) were injected subcutaneously into the shoulder of nu/nu mice, and tumor weight was examined after 2 weeks. Likewise, tumor weight for the cells expressing wild *MAP2K1*, R47Q, R49L or I204T mutant was examined after 3 weeks and that for cells expressing wild *MAP2K1* or the P306H mutant was examined after 4 weeks. Data are means \pm SD of values from four injection sites. The difference in tumor size is evaluated by Student's *t*-test.

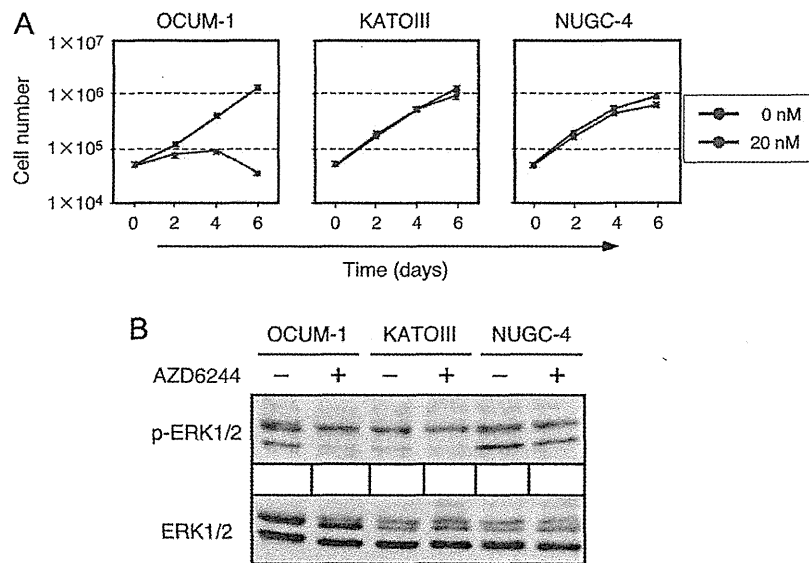


Fig. 2. MAP2K1(Q56P)-dependent growth of OCUM-1 cells. (A) OCUM-1, KATOIII and NUGC-4 cells were cultured in the absence or presence of AZD6244 (20 nM), and cell number was determined at the indicated times. Data are means \pm SD from three separate experiments. (B) Total cell lysates were obtained from each cell line treated with (+) or without (-) 400 nM of AZD6244 for 24 h and probed with antibodies to phosphorylated (p-) or total forms of ERK1/2.

paired-end adaptors used for PCR amplification of fragmented *MAP2K1* cDNAs to be analyzed by a GAIx sequencer (Figure 3A). The first two bases in each GAIx read sequence thus correspond to the tag. The diversity of the tag sequences allows a total of 16 independent samples to be analyzed together in a single lane of an Illumina flow cell.

We amplified the entire coding region of *MAP2K1* cDNA from a total of 171 cell lines or clinical specimens of human epithelial cancer, including 26 samples of esophageal cancer (14 cell lines and 12 specimens), 88 samples of gastric cancer (2 cell lines, 46 specimens with a pathological classification of poorly differentiated cell morphology and 40 specimens with well-differentiated cell morphology), 13 samples of breast cancer (2 cell lines and 11 specimens) and 44 specimens of colon cancer. The resulting cDNAs were then subjected to multiplex deep sequencing with four to six different samples (with different tags) run in the same lanes.

A BLAST search of non-synonymous substitutions in the *MAP2K1* sequence revealed an additional five amino acid changes (2.9%) in our 171 samples: I204T in a specimen of poorly differentiated gastric cancer (scirrhous-type), R47Q in a specimen of breast cancer and R49L, D67N and P306H in different specimens of colon cancer (Figure 3B; Supplementary Table 1 is available at *Carcinogenesis* Online). The proportion of mutant reads within the total coverage varied substantially among specimens. For instance, *MAP2K1* reads containing a G-to-A substitution at nucleotide position 140 for the R47Q change in breast cancer occupied ~94% of total reads at this position, suggesting a loss of heterozygosity at the *MAP2K1* locus. In contrast, the reads containing a T-to-C substitution for the I204T change occupied only 7.9% of the total reads from the specimen of scirrhous-type gastric cancer, confirming the low percentage of tumor cells in a given specimen for this highly infiltrative cancer.

We next examined whether these point mutations were somatic alterations by sequencing genomic DNA for the corresponding regions prepared from the tumor as well as paired non-tumor tissue. Unfortunately, an extra specimen for the breast cancer patient (MMK5) was not available, and so we were not able to confirm the genomic change responsible for the R47Q mutation. For this patient, however, the G-to-A change was readily confirmed in cDNA sequencing by the Sanger method (data not shown). For the other four mutations, we did confirm cancer-specific sequence alterations in genomic DNA (Figure 3C). We thus identified a total of six amino acid

substitutions in *MAP2K1*, two (Q56P and D67N) of which had been described previously and had their transforming ability confirmed (16,18), but the remaining four were novel.

Transforming ability of mutant *MAP2K1* proteins

To assess the transforming potential of the novel *MAP2K1* mutations, we constructed recombinant retroviruses encoding each mutant in order to infect mouse 3T3 fibroblasts. Incubation of the cells for 13 days led to the formation of multiple transformed foci with each mutant but not with wild-type *MAP2K1* (Figure 1A). As in the case for the Q56P mutant, no transformed foci were detected for the kinase-dead forms (K97M) or with the incubation with AZD6244. As demonstrated in Figure 1A, a sustained treatment with the compound induced marked cell death among the 3T3 cells except for those expressing the Q56P mutant. Furthermore, it should be noted that the number of transformed foci expressing the R47Q or P306H mutant was substantially fewer than that of cells expressing the other mutants.

Such 3T3 cells were also injected into the shoulder of nu/nu mice (Figure 1B). At 3 weeks after the injection, all of the R47Q, R49L and I204T mutants but not wild-type *MAP2K1* or the P306H mutant, generated palpable tumors at every injection site. These data further confirmed the transforming potential of the R47Q, R49L and I204T mutants in addition to Q56P. At 4 weeks after the 3T3 injection, we could identify subcutaneous tumors for the P306H, but the cells expressing wild-type *MAP2K1* generated small tumors at several sites as well. Therefore, the transforming potential for *MAP2K1*(P306H) may be modest.

To examine the effect of the *MAP2K1* mutants on the activation status of the downstream kinases ERK1/2, we introduced *MAP2K1* or its mutants into HEK293 cells and evaluated the phosphorylation level of ERK1/2 by immunoblot analysis (Figure 3D). Expression of wild-type *MAP2K1* resulted in only a small increase in the level of phosphorylation (activation) of ERK1/2. In contrast, *MAP2K1*(Q56P) induced a marked increase in ERK1/2 activity, whereas a kinase-dead form of this mutant had largely lost this ability, suggesting that *MAP2K1*(Q56P) activates ERK1/2 in a manner dependent on its enzymatic activity. All of the other mutants tested (R47Q, R49L, I204T and P306H) also markedly increased the level of ERK1/2 phosphorylation, further confirming their activating potential.

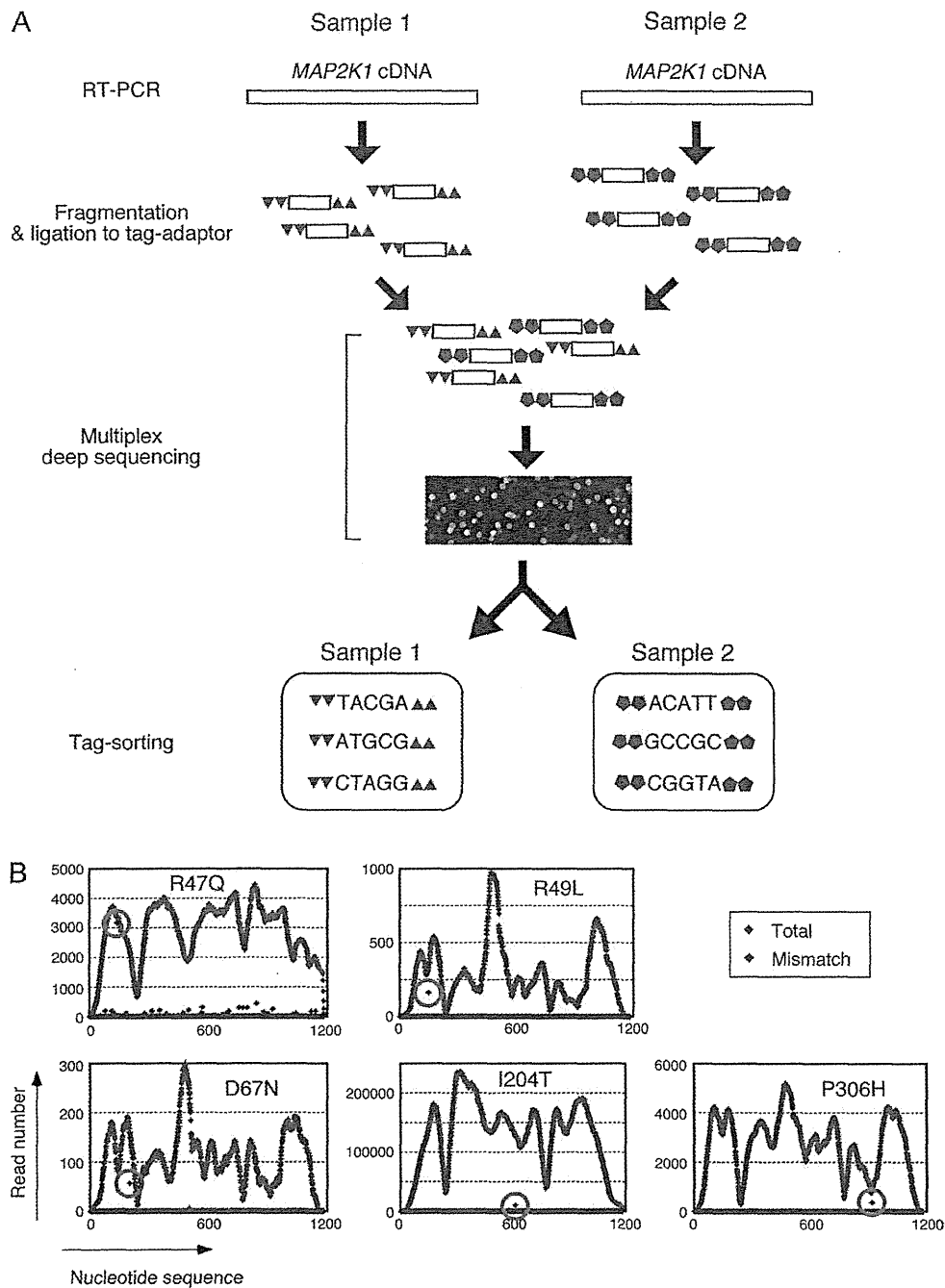


Fig. 3. Multiplex deep sequencing of *MAP2K1* cDNAs. (A) *MAP2K1* cDNA was synthesized from 171 cell lines or clinical specimens, fragmented and ligated to sample-specific two-base-tagged adaptors. The cDNA fragments with different two-base tags were mixed and subjected to deep sequencing in the same lane of an Illumina flow cell. The resultant read sequences were sorted, with the two-base tags used as an identification number for each sample. (B) *MAP2K1* cDNAs obtained from five cancer specimens harboring the R47Q, R49L, D67N, I204T or P306H mutations were sequenced with the GAIIX system. The numbers for total read coverage (Total) and mismatched reads (Mismatch) are shown at each position of the cDNAs with blue and red diamonds, respectively. The position for mismatched reads for each *MAP2K1* mutation is indicated by a green circle. (C) Genomic DNA corresponding to R49, D67, I204 or P306 positions of *MAP2K1* was amplified by PCR from tumor and paired normal tissue specimens and was then subjected to Sanger sequencing. Mutated bases are shown in red letters. The PCR product for the tumor harboring the I204T mutation was cloned into a plasmid vector before Sanger sequencing. (D) Expression vectors for *MAP2K1* (Wild), *MAP2K1*(Q56P), the kinase-dead mutant *MAP2K1*(Q56P/K97M), *MAP2K1*(R47Q), *MAP2K1*(R49L), *MAP2K1*(I204T) or *MAP2K1*(P306H) or the corresponding empty vector (-), were introduced into HEK293 cells. Lysates of the transfected cells were subjected to immunoblot analysis with antibodies to phosphorylated (p-) or total forms of ERK1/2.

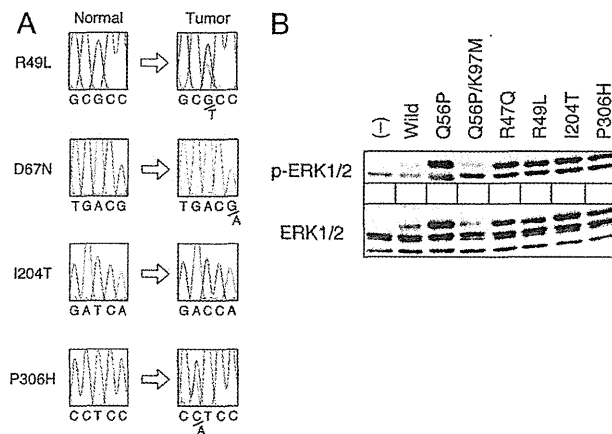


Fig. 3. continued.

Discussion

We have identified the Q56P mutant of MAP2K1 in a cell line of scirrhous-type gastric cancer and have shown that these cancer cells are dependent on the mutated MAP2K1 for growth. Furthermore, deep sequencing of *MAP2K1* cDNAs derived from 171 cancer cell lines or clinical specimens revealed one known and four novel mutations of MAP2K1, all of which possess transforming potential. A total of six (3.5%) distinct activating mutations were thus discovered for MAP2K1 in our cohort of 172 cell lines and clinical specimens (Figure 4).

In addition to MAP2K1 mutations identified in individuals with congenital cardiofaciocutaneous syndrome (19), MAP2K1 mutations have been detected in various cancers, albeit infrequently (20). The D67N substitution was discovered in 1 of 15 ovarian cancer cell lines (18). On the other hand, two cases of NSCLC harboring a somatic K57N mutation of MAP2K1 were detected among 207 primary tumor specimens, whereas the Q56P change was identified in 1 of 85 NSCLC cell lines (16). Furthermore, two cases of colorectal cancer harboring a somatic amino acid change (R201H or E203K) of MAP2K1 were detected by screening of 55 cases, but none of 38 cases of breast cancer was found to be associated with a MAP2K1 mutation (21); the same study also found MAP2K1(Y134C) in one cancer cell line. Whereas the Q56P, K57N and D67N substitutions are known to activate MAP2K1, it remains unclear whether these other amino acid changes affect MAP2K1 activity. These previous data, together with our present results, thus suggest that MAP2K1 is infrequently activated by point mutations in various epithelial tumors.

Interestingly, the transforming potential of the MAP2K1 mutants did not correlate always with the phosphorylation level of their downstream targets ERK1/2. While all of R47Q, R49L, Q56P, I204T and P306H mutations similarly induced phosphorylation of ERK1/2 in HEK293 cells (Figure 3D), their transforming potential as judged by 3T3 cells substantially differs (Figure 1). Furthermore, mouse 3T3 cells have a high level of ERK1/2 phosphorylation, and introduction of MAP2K1 mutants only slightly affect the phosphorylation level (data not shown). These data may imply that MAP2K1-dependent transformation of 3T3 cells is, at least, partially mediated through ERK1/2-independent pathways as demonstrated previously (22) or is cell context-dependent.

Known and novel MAP2K1 mutations appear to cluster in two hotspots (Figure 4): a hinge region (R47, R49, Q56, K57 and D67) between the coiled-coil and catalytic domains and the activation loop of the kinase domain (R201, E203 and I204). Amino acid substitutions in the activation loop frequently increase enzymatic activity in a wide variety of protein kinases (23). With regard to the hinge region, artificial truncation of the coiled-coil domain was shown previously to result in constitutive activation of MAP2K1 (24). It is thus likely that

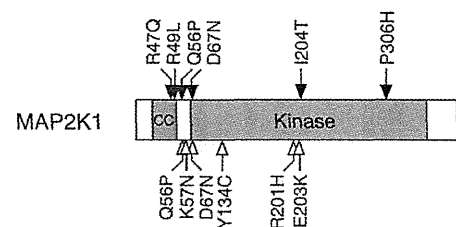


Fig. 4. Schematic representation of the protein structure of MAP2K1 showing amino acid substitutions identified in the present study (closed arrows) and in previous studies (open arrows). CC, coiled-coil domain.

the coiled-coil domain of MAP2K1 negatively regulates enzymatic activity and that mutations that affect the proper structure or function of the coiled-coil domain may release the enzymatic activity of MAP2K1 from such inhibition.

In our study, we took advantage of multiplex deep sequencing to screen for mutations of *MAP2K1* cDNA in a sensitive manner, allowing interrogation of the target cDNA at a high level of coverage among hundreds of samples. Such deep sequencing is likely to be especially important for analysis of specimens of infiltrative tumors or of tumors with large amounts of necrotic tissue, as shown by the detection of the I204T mutation as a low proportion of reads for a specimen of scirrhous-type gastric cancer. Likewise, from pleural effusion of a patient with NSCLC positive for EML4-ALK, we detected a small fraction of tumor cells with acquired mutations responsible for resistance to an ALK inhibitor (25). Such an approach should prove useful for the detection of *MAP2K1* mutations in other types of cancer, and it potentially constitutes a relatively simple, yet highly sensitive, approach to the screening of any target gene or cDNA for nucleotide changes among hundreds of clinical specimens.

Supplementary material

Supplementary Table 1 and Figure 1 can be found at <http://carcin.oxfordjournals.org/>.

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Ex Vivo Expansion of Human CD8⁺ T Cells Using Autologous CD4⁺ T Cell Help

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Abstract

Background: Using *in vivo* mouse models, the mechanisms of CD4⁺ T cell help have been intensively investigated. However, a mechanistic analysis of human CD4⁺ T cell help is largely lacking. Our goal was to elucidate the mechanisms of human CD4⁺ T cell help of CD8⁺ T cell proliferation using a novel *in vitro* model.

Methods/Principal Findings: We developed a genetically engineered novel human cell-based artificial APC, aAPC/mOKT3, which expresses a membranous form of the anti-CD3 monoclonal antibody OKT3 as well as other immune accessory molecules. Without requiring the addition of allogeneic feeder cells, aAPC/mOKT3 enabled the expansion of both peripheral and tumor-infiltrating T cells, regardless of HLA-restriction. Stimulation with aAPC/mOKT3 did not expand Foxp3⁺ regulatory T cells, and expanded tumor infiltrating lymphocytes predominantly secreted Th1-type cytokines, interferon- γ and IL-2. In this aAPC-based system, the presence of autologous CD4⁺ T cells was associated with significantly improved CD8⁺ T cell expansion *in vitro*. The CD4⁺ T cell derived cytokines IL-2 and IL-21 were necessary but not sufficient for this effect. However, CD4⁺ T cell help of CD8⁺ T cell proliferation was partially recapitulated by both adding IL-2/IL-21 and by upregulation of IL-21 receptor on CD8⁺ T cells.

Conclusions: We have developed an *in vitro* model that advances our understanding of the immunobiology of human CD4⁺ T cell help of CD8⁺ T cells. Our data suggests that human CD4⁺ T cell help can be leveraged to expand CD8⁺ T cells *in vitro*.

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Competing Interests: MOB, LMN and NH have filed a patent application related to aAPC/A2. The patent application number is 10/850,294 and is entitled, "Modified Antigen-Presenting Cells." The authors confirm that this application does not alter their adherence to all PLoS ONE policies on the sharing of data and materials.

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Introduction

It is now well accepted that neoplastic cells are immunogenic and that tumors develop in the context of immune recognition by the host [1,2]. Tumor-associated antigens that serve as immune targets include cell lineage differentiation antigens, cancer-testes antigens, and neoantigens produced by mutations in the cancer cell's unstable genome. Mutational events can give rise to multiple immunogenic MHC class I and II restricted, non-self epitopes capable of inducing strong immune responses to the tumor [3,4]. In several malignancies, anti-tumor T cell responses, with infiltration of tumors by CD8⁺ T lymphocytes and local production of interferon- γ and IL-2, have been associated with improved clinical prognosis [5–8].

Counter regulatory immune responses, however, also develop in the cancer-bearing host. Tumors subvert the immune response by

secreting chemotactic factors that recruit immune suppressive elements, thereby inhibiting the function of anti-tumor effectors [9]. Tumor infiltration by T regulatory (Treg) cells has been correlated with inferior clinical outcomes in several tumors [10,11]. These findings have led to the proposal that immune recognition of cancer involves the balancing of opposing forces: anti-tumor effectors vs. pro-tumor regulatory elements [10,12,13]. In fact, a high ratio of Treg cells to CD8⁺ T cells within the tumor microenvironment has been associated with poorer survival [14,15].

Adoptive T cell therapy is a promising treatment modality designed to amplify the anti-tumor immune response. Anti-tumor effectors are expanded *in vitro*, away from the pro-tumor milieu of the cancer bearing host, and then reinfused as a cellular therapy [16–21]. Successful approaches showing clinical activity include adoptive transfer of tumor antigen-specific T cell lines or clones

that have been derived from the peripheral blood. Specificity can be achieved by stimulating antigen-specific precursor T cells or through genetic modification of expanded bulk T cells to express cloned or chimeric T cell receptor (TCR) genes [22–26]. Alternatively, the nascent, endogenous immune effector response to the tumor can be amplified by expanding tumor-infiltrating lymphocytes (TIL) *in vitro*. Adoptive cell transfer of *in vitro* activated TIL has achieved major clinical responses when patients first undergo lymphodepletion and are then given high dose IL-2 after adoptive transfer [17,27]. Lymphodepletion augments the persistence and function of transferred TIL not only by reducing or temporarily eliminating Treg cells, but also by reducing cytokine sinks that results in the accumulation of homeostatic cytokines such as IL-7 and IL-15 [28,29].

The optimal method for generating clinically effective T cell grafts *in vitro* has yet to be established [21,30]. In order to achieve massive numerical expansion of T cells, current methods necessitate the use of soluble monoclonal antibodies (mAb), allogeneic feeder PBMC, EBV transformed lymphoblastoid cell lines, and/or undefined culture supernatants. Consequently, these requirements present formidable challenges and costs that prevent the widespread clinical application of this therapy. While adoptive transfer of anti-tumor CD4⁺ T cells can be efficacious, expansion of anti-tumor CD8⁺ T cells is also an important goal, particularly in light of the association between their persistence and clinical responses [18,31–33].

Insights into requirements for augmenting the expansion of both CD4⁺ and CD8⁺ T cells will help further improve methods to generate T cell grafts for adoptive therapy. CD4⁺ T cells help generate effective immune responses by sustaining CD8⁺ T cell proliferation, preventing exhaustion, and establishing long-lived functional memory [34]. In mouse models, common γ -chain receptor cytokine and CD40 signaling can mediate CD4⁺ T cell help [34–44]. In clinical studies, CD4⁺ T cells have also been implicated in promoting the persistence and anti-tumor activity of antigen-specific CD8⁺ T cells in patients [45,46]. However, the mechanisms of human CD4⁺ T cell help are less well understood. To conduct a mechanistic analysis of human CD4⁺ T cell help, we developed a novel, human cell-based aAPC, aAPC/mOKT3, which induces both CD4⁺ and CD8⁺ T cell expansion without allogeneic feeder cells. The removal of allogeneic feeder cells from our T cell culture system enabled us to precisely isolate molecules mediating help of CD8⁺ T cell expansion that are expressed or secreted by human CD4⁺ T cells.

Results

K562-based aAPC expressing membranous OKT3 induces CD3⁺ T cell expansion

We and others have previously reported the generation of aAPC derived from the human erythroleukemia cell line K562 [47–51]. K562 serves as an excellent platform for generating aAPC since it expresses no HLA class I or II molecules, but highly expresses adhesion molecules such as CD54 and CD58. Using K562, we developed a novel aAPC, aAPC/mOKT3, capable of expanding CD3⁺ T cells regardless of HLA subtype (Figure 1A, Figure S1). This aAPC was engineered to express a membranous form of the anti-CD3 mAb, OKT3, on its cell surface, thus obviating the need for adding soluble mAb to T cell cultures or loading it onto aAPC as described elsewhere [51,52]. aAPC/mOKT3 also ectopically expresses immunostimulatory molecules CD80 and CD83. We and others have shown that CD83 delivers a CD80 dependent signal that promotes lymphocyte longevity [47,53,54].

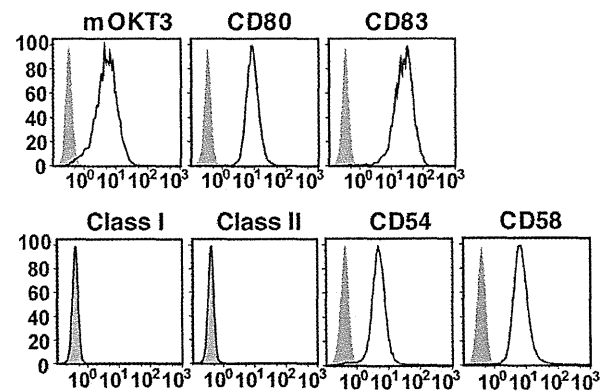


Figure 1. Generation of aAPC/mOKT3. Surface expression of a transduced membranous form of anti-CD3 mAb, and transduced CD80, CD83, and endogenous HLA class I, class II, CD54, and CD58 on aAPC/mOKT3 is shown. A membranous form of anti-CD3 mAb on aAPC/mOKT3 (open) and wild type K562 (shaded) was stained using goat anti-mouse IgG (H+L). Other surface molecules were stained with each specific mAb (open) and isotype control (shaded) and analyzed by flow cytometry. Note the lack of endogenous expression of HLA class I and II on aAPC/mOKT3. doi:10.1371/journal.pone.0030229.g001

Stimulation of CD3⁺ T cells with aAPC/mOKT3 induces robust CD8⁺ T cell expansion

Peripheral CD3⁺ T cells expanded with aAPC/mOKT3 were phenotypically characterized after 28 days in culture (Figure 2). While the number of both CD4⁺ and CD8⁺ T cells increased, CD8⁺ T cells expanded substantially better than CD4⁺ T cells, and therefore dominated cultures from every donor tested (Figure 2A). This is in contrast to other pan T cell expansion systems such as anti-CD3/CD28 mAb-coated beads, which invariably favor the expansion CD4⁺ T cells over CD8⁺ T cells [55] (Figure 2B). Similar fold expansion of CD3⁺ T cells was obtained with the aAPC/mOKT3-based and antibody-coated bead-based expansion systems. T cells expanded using aAPC/mOKT3 displayed a central memory~effector memory phenotype (CD45RA⁺ CD54RO⁺ CD62L^{+/−}) and retained expression of receptors for IL-2, IL-7, and IL-21 (Figure 2C). CD40 ligand was highly expressed by CD4⁺ T cells but not CD8⁺ T cells. Importantly, expanded CD4⁺ CD25⁺ T cells did not express Foxp3, indicating that immunoinhibitory Treg cells did not proliferate well (Figure 2D).

aAPC/mOKT3 induces unbiased CD3⁺ T cell expansion, preserving the repertoire for viral and tumor-associated antigens

In order to evaluate whether stimulation with aAPC/mOKT3 induced broad expansion of CD3⁺ T cells, TCR V β repertoire analysis was performed. No obvious skewing in the TCR V β usage of both CD4⁺ and CD8⁺ T cell populations was revealed, supporting “unbiased” T cell expansion by aAPC/mOKT3 (Figure 3A). Moreover, HLA-restricted antigen-specific CD8⁺ cytotoxic T lymphocytes (CTL) against viral and tumor antigens could be generated from CD3⁺ T cells initially expanded for four weeks using aAPC/mOKT3 (Figure 3B and 3C). The functional avidity of these tumor antigen-specific T cells was sufficient to recognize tumor targets endogenously expressing antigen, confirming that the T cell repertoire for tumor antigen recognition was preserved (Figure 3C). We also confirmed that stimulation with aAPC/mOKT3 induced the expansion of tumor-antigen specific T cells. After 28 days in culture, MART1 peptide specific CD8⁺ T cell expansion was 420–1,150 fold (Figure S1D).

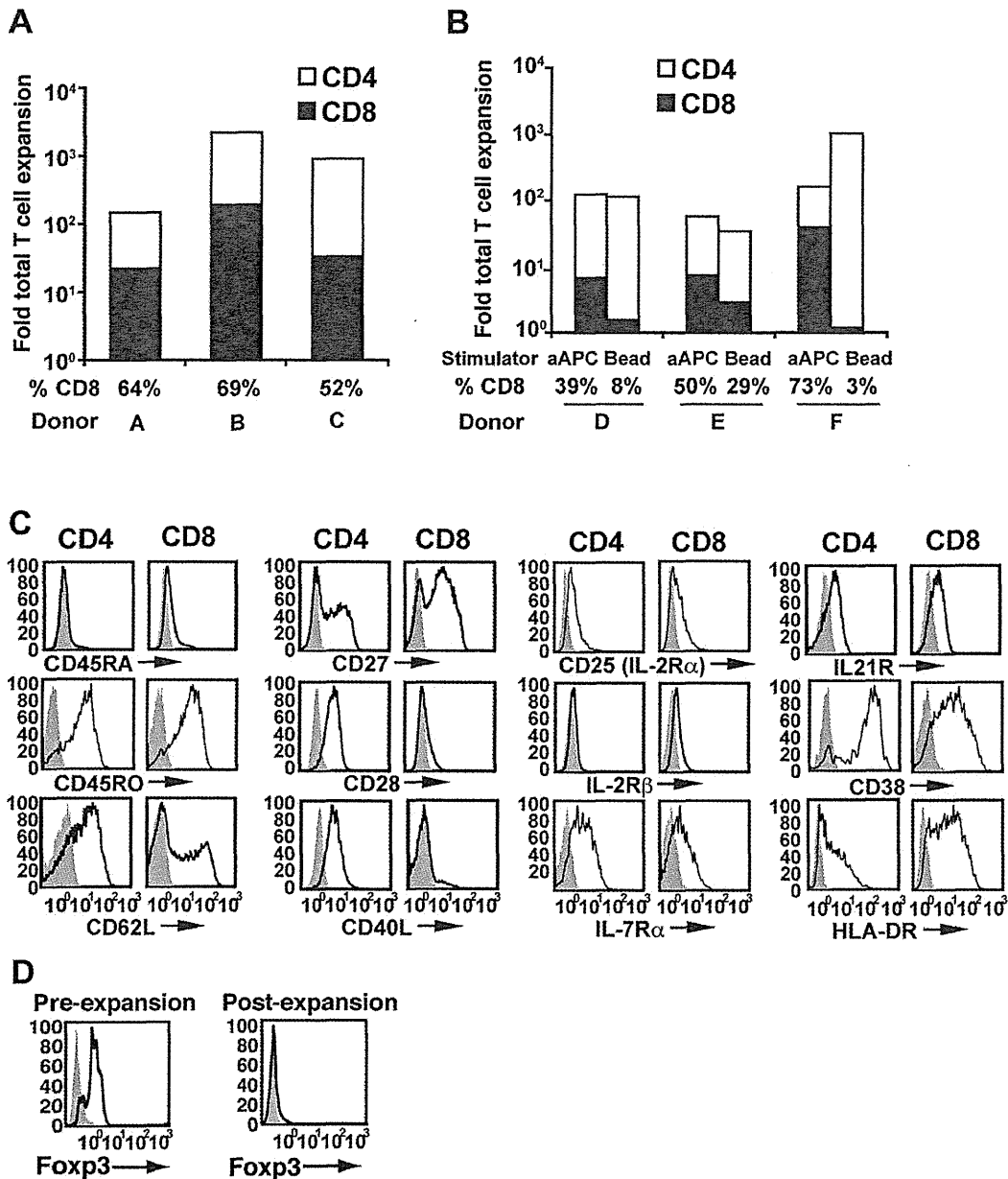


Figure 2. aAPC/mOKT3 expands both CD4⁺ and CD8⁺ T cells without using allogeneic feeder PBMC. (A) CD3⁺ T cells were stimulated twice with aAPC/mOKT3 and supplemented with IL-2 between stimulations. Fold expansion of CD3⁺ T cells over one month is shown for three donors. Shading shows the proportion of expanded CD4⁺ (white) and CD8⁺ (black) T cells, and percent CD8⁺ T cells is indicated. (B) CD3⁺ T cells were stimulated twice with aAPC/mOKT3 or beads (Dynabeads CD3/CD28) and supplemented with IL-2 between stimulations. Fold expansion of CD3⁺ T cells over one month is shown for three donors. Shading shows the proportion of expanded CD4⁺ (white) and CD8⁺ (black) T cells, and percent CD8⁺ T cells is indicated. (C) CD3⁺ T cells were expanded as described in Figure 2A. Expression of surface molecules on gated CD4⁺ and CD8⁺ T cells is shown (open). Isotype mAb staining was used as a control (shaded). (D) CD4⁺ CD25⁺ Foxp3⁺ Treg cells, present pre-expansion, were absent in expanded cultures. CD4⁺ CD25⁺ cells, pre- and post-expansion, were stained intracellularly with anti-Foxp3 mAb (open) and isotype control (shaded). doi:10.1371/journal.pone.0030229.g002

aAPC/mOKT3 expands functional TIL but not contaminating Treg cells

Using aAPC/mOKT3, lymphocytes derived from malignant ascites (breast and ovarian cancer) and melanoma metastases were successfully expanded without adding any allogeneic feeder cells (Figure 4A). As observed with peripheral CD3⁺ T cells in Figure 2A, CD8⁺ T cells predominantly expanded in all

cultures, including those that initially contained a minimal percentage of CD8⁺ T cells. Importantly, Foxp3⁺ cells did not proliferate well (Figure 4B). As with peripheral CD3⁺ T cells, expanded TIL had a central memory~effector memory phenotype (CD45RA⁻ CD62L^{+/+}) consistent with a lack of terminal differentiation (Figure S2). Furthermore, expanded T cells highly expressed CD27 and CD28 which are associated

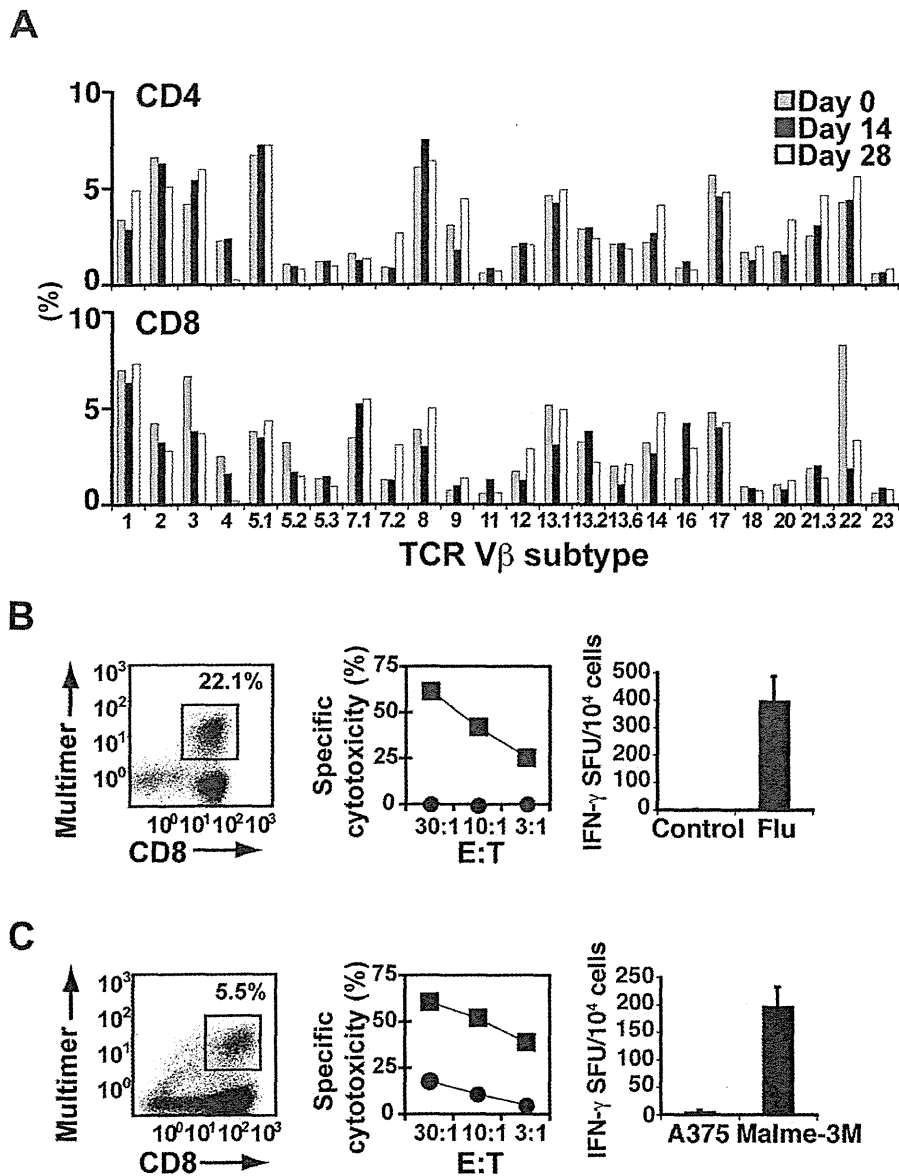


Figure 3. Expansion with aAPC/mOKT3 does not induce skewing of the TCR V β repertoire. (A) TCR V β subfamily analysis before and after stimulation with aAPC/mOKT3 is shown. CD3⁺ T cells were stimulated with aAPC/mOKT3 on days 0 and 14 and were treated with IL-2 at 300 IU/ml between stimulations. TCR V β usage analysis was performed on days 0, 14, 28. Data shown is on gated CD4⁺ and CD8⁺ T cells. (B, C) A2⁺ CD3⁺ T cells were stimulated twice with aAPC/mOKT3 for one month. Subsequently, CD8⁺ T cells were purified from expanded CD3⁺ T cells and further stimulated with aAPC/A2 pulsed with Flu or MART1 peptide. (B) Flu specificity was demonstrated by multimer staining (left). Functional competence was demonstrated by antigen-specific cytotoxicity (middle) and IFN- γ secretion (right). T2 cells pulsed with Flu peptide (■) or control peptide (●) were used as targets. (C) MART1 specificity was similarly demonstrated by multimer staining (left). The HLA-A2⁺/MART1⁺ melanoma line, Malme-3M (■), and the HLA-A2⁺/MART1⁻ melanoma line, A375 (●), were used as targets in cytotoxicity (middle) and IFN- γ ELISPOT assays (right). doi:10.1371/journal.pone.0030229.g003

with T cell survival and persistence *in vivo* [56-59]. They also secreted high quantities of IFN- γ and IL-2, while IL-4 secretion was lower and no IL-10 was produced (Figure 4C). These results demonstrate that the aAPC/mOKT3-based system can expand tumor-infiltrating CD8⁺ T cells in the presence of autologous CD4⁺ T cells, and that they display phenotypic and functional characteristics consistent with central memory~effector memory T cells.

IL-2 and IL-21 are necessary, but not sufficient, for CD4⁺ T cell-mediated help of CD8⁺ T cell expansion

Using the aAPC/mOKT3-based expansion system, we compared the expansion of CD8⁺ T cells in the presence or absence of CD4⁺ T cells. CD8⁺ T cells expanded much better in the presence of CD4⁺ T cells (Figure 5A), suggesting the presence of CD4⁺ T cell help for CD8⁺ T cells in these aAPC/mOKT3-based cultures. We tested whether this "help" was mediated by soluble factors or

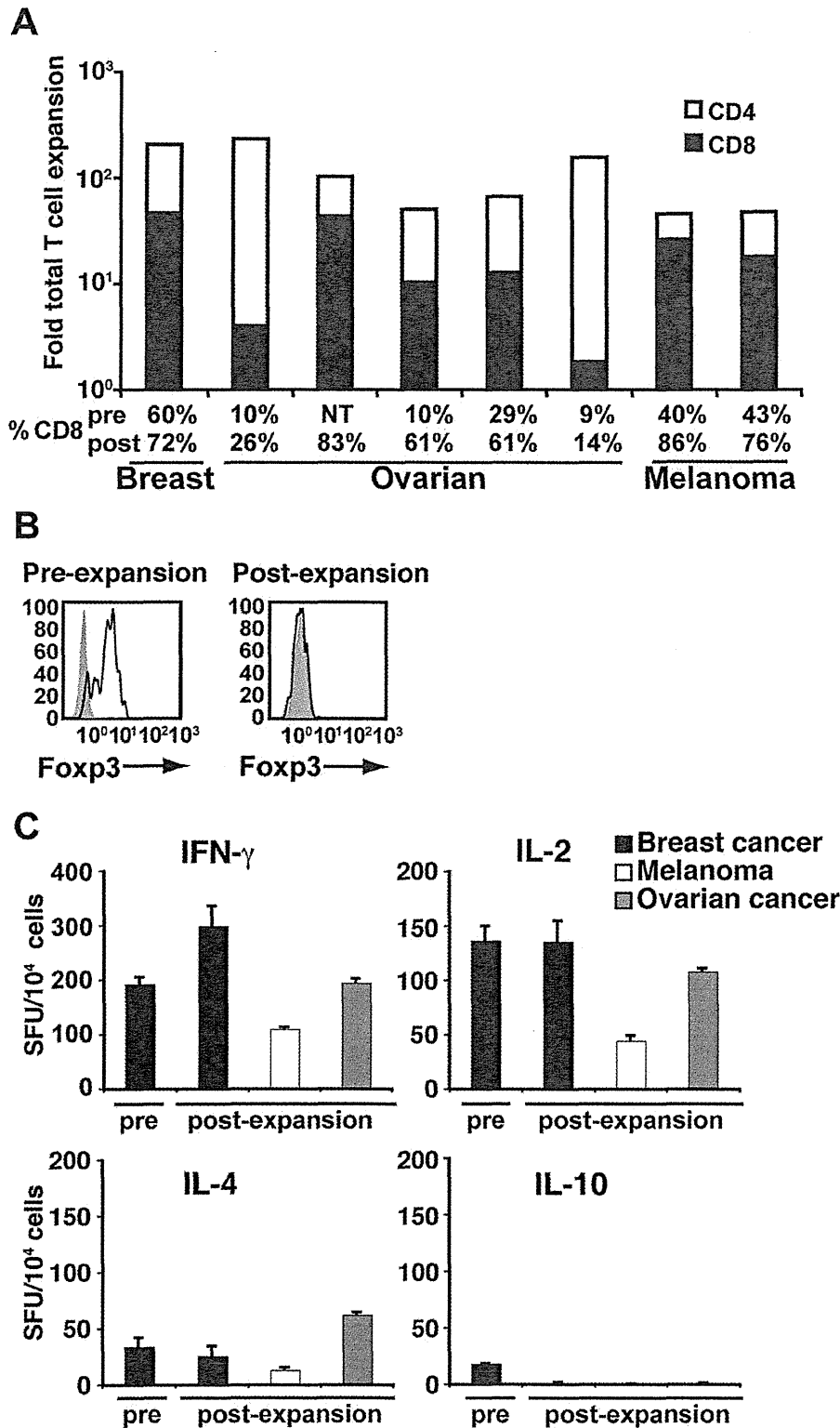


Figure 4. aAPC/mOKT3 expanded TIL are Foxp3 negative and secrete predominantly Th1 cytokines. (A) Expansion of TIL obtained from breast and ovarian cancer ascites and melanoma metastases is shown. Shading indicates the proportion of CD4⁺ (white) and CD8⁺ (black) T cells in expanded cultures. The percentage of CD8⁺ T cells in pre- and post-expansion cultures is shown. Note that in all samples tested, the percentage of CD8⁺ T cells increased even in those that initially contained a minimal percentage of CD8⁺ T cells. NT denotes not tested. (B) CD4⁺ CD25⁺ Foxp3⁺ Treg

cells, present pre-expansion, were not detectable after one month of culture. CD4⁺ CD25⁺ cells were intracellularly stained with anti-Foxp3 mAb (open) and isotype control (shaded). (C) IFN- γ , IL-2, IL-4, and IL-10 secretion of expanded TIL was determined by ELISPOT assays. Cytokine secretion by TIL from the breast cancer ascites specimen prior to expansion is shown as a control. Pre-expansion samples from melanoma and ovarian cancer specimens were not studied because of low initial cell numbers.
doi:10.1371/journal.pone.0030229.g004

cell-cell contact using the transwell assay (Figure 5B). A single stimulation, without any exogenously added cytokines, expanded CD8⁺ T cells by an average of 40.5% better when CD4⁺ T cells were present but separated from CD8⁺ T cells by the transwell membrane ($P < 0.005$). In co-cultures where CD4⁺ and CD8⁺ T cells were mixed, allowing for direct cell-cell contact, CD8⁺ T cells expanded more than in cultures where they were separated from CD4⁺ T cells by the transwell membrane ($P < 0.05$). These results suggest that observed CD4⁺ T cell help involves both soluble factors and cell-cell contact.

To identify molecules mediating the observed CD4⁺ T cell help, culture supernatants of CD4⁺/CD8⁺ T cell mixed and separate cultures were tested for a panel of soluble factors (Figure 5C and Table S1). Greater quantities of MIP-1 α , MIP-1 β , and RANTES were detected in CD4⁺/CD8⁺ T cell mixed cultures compared to separate cultures, suggesting increased production in mixed cultures. In contrast, IL-2 and IL-21, as well as IL-10, IL-17, TNF- α , and TNF- β , were detected at lower levels in mixed cultures, consistent with more consumption or less production of these cytokines.

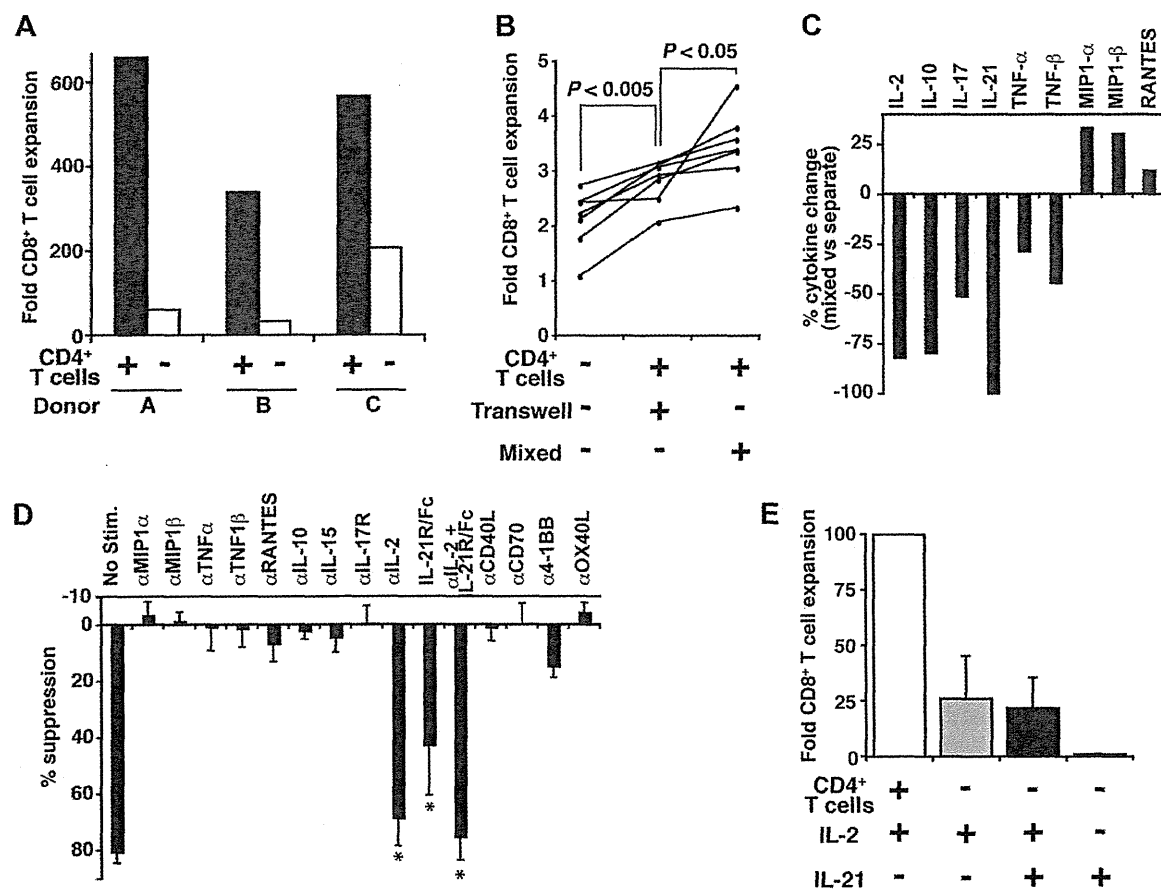


Figure 5. Autologous CD4⁺ T cell secretion of IL-2/IL-21 is necessary but not sufficient to help CD8⁺ T cells proliferate. (A) CD8⁺ T cells were stimulated twice by aAPC/mOKT3 with or without CD4⁺ T cells and treated with IL-2 between stimulations. Fold expansion of CD8⁺ T cells over 28 days is shown for 3 donors. (B) CD8⁺ T cells were stimulated only once by aAPC/mOKT3 with or without CD4⁺ T cells in transwell plates. No IL-2 or other cytokines were given. Fold expansion of CD8⁺ T cells over 6 days is shown for 7 donors. (C) Culture supernatants were tested for a panel of soluble factors to identify mediators of CD4⁺ T cell help. Relative changes in cytokines, comparing mixed vs. separate cultures, are shown. Data is representative of two donors. Absolute values for two donors are shown in Table S1. (D) Suppression of CD8⁺ T cell expansion in the presence of CD4⁺ T cells by blocking reagents is presented as percent suppression relative to control. Values indicate mean of four independent experiments; error bars show s.d. * $P < 0.005$. (E) CD8⁺ T cells were stimulated twice with aAPC/mOKT3 in the presence or absence of CD4⁺ T cells. IL-2, IL-21, or both were added in each condition. Fold expansion of CD8⁺ T cells over 28 days is shown. Percent expansion was calculated by dividing the number of expanded CD8⁺ T cells by the number of CD8⁺ T cells expanded in the presence of CD4⁺ T cells. Values indicate mean of six independent experiments; error bars show s.d.
doi:10.1371/journal.pone.0030229.g005

To differentiate between “more consumption” and “less production,” CD4⁺/CD8⁺ T cell mixed cultures were stimulated in the presence of blocking reagents, and suppression of CD8⁺ T cell expansion was assessed (Figure 5D). Blockade of IL-2 and IL-21 resulted in a reduction of expansion by 68.8% ($P < 0.005$) and 42.9% ($P < 0.005$), respectively. These results indicate that the decreased levels of IL-2 and IL-21 in CD4⁺/CD8⁺ T cell mixed cultures were due to more consumption rather than less production and that these cytokines may be necessary mediators of CD4⁺ T cell help in this human-based *in vitro* system. To test whether IL-2/IL-21 could substitute for the observed CD4⁺ T cell help, CD8⁺ T cells stimulated with aAPC/mOKT3 were supplemented with IL-2, IL-21, or both (Figure 5E). CD8⁺ T cells did not expand without IL-2. The addition of IL-2 with or without IL-21 did not improve CD8⁺ T cell expansion to the level observed when cocultured with CD4⁺ T cells, demonstrating that IL-2 plus IL-21 are not sufficient to replace CD4⁺ T cell help.

Exogenous IL-2/IL-21 and upregulation of IL-21 receptor can partially recapitulate CD4⁺ T cell help of CD8⁺ T cell expansion *in vitro*

Interestingly, we observed that higher expression of the IL-21 receptor (IL-21R) on CD8⁺ T cells occurred when CD4⁺ T cells were present during stimulation by aAPC/mOKT3 (Figure 6A).

Higher IL-21R expression on CD8⁺ T cells was not induced by supplementing cultures with IL-2 and IL-21 (data not shown). This prompted us to hypothesize that increased upregulation of IL-21R on CD8⁺ T cells is critical for the full effect of IL-21 secreted by CD4⁺ T cells. We constitutively expressed IL-21R on CD8⁺ T cells (Figure 6B, left) and stimulated them with aAPC/mOKT3 in the presence of IL-2/IL-21. In accordance with the transduction efficiency of IL-21R to 75.9%, CD8⁺ T cell proliferation partially increased to levels seen in the presence of CD4⁺ T cells (Figure 6B, right). This indicates that elevated expression of IL-21R is necessary and can partially recapitulate CD4⁺ T cell help for CD8⁺ T cell proliferation.

Discussion

A novel human cell-based aAPC expanded CD3⁺ T cells *in vitro* without the addition of allogeneic feeder PBMC. Phenotypic analysis of expanded healthy donor T cells and TIL showed, that while both CD4⁺ and CD8⁺ T cells expanded, CD8⁺ T cells predominated. In this model system, we demonstrated that CD8⁺ T cell expansion depended on the presence of CD4⁺ T cells, suggesting that CD4⁺ T cells provided help to proliferating CD8⁺ T cells. The CD4⁺ T cell secreted cytokines, IL-2 and IL-21, and the CD4⁺ T cell-dependent upregulation of IL-21R on CD8⁺ T cells were necessary for the observed CD4⁺ T cell help.

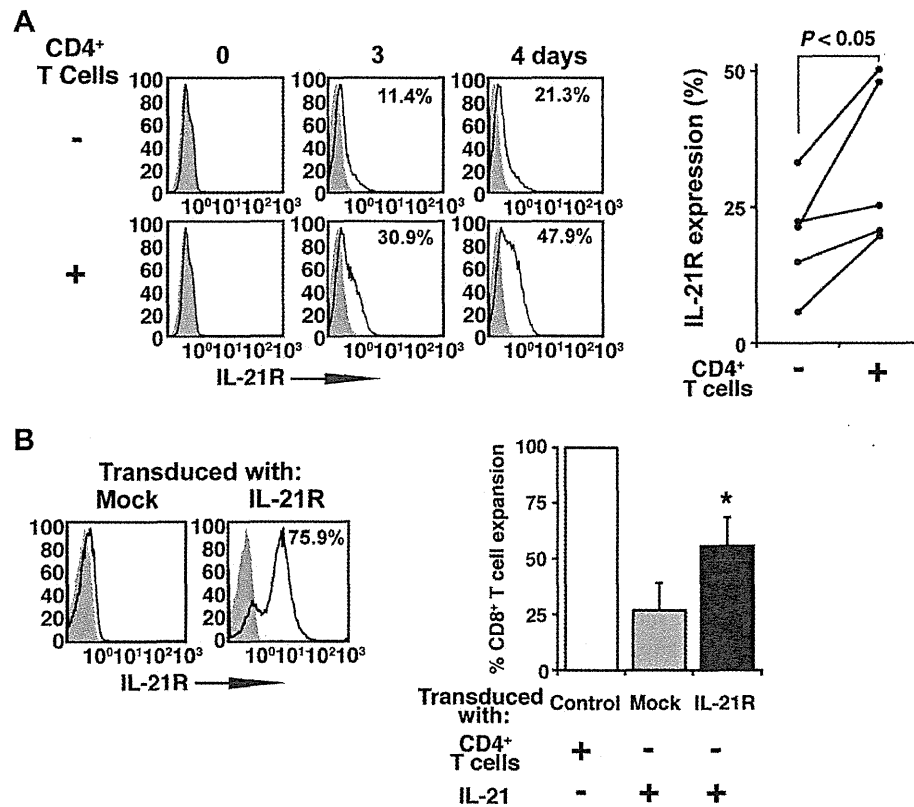


Figure 6. IL-2/IL-21 and upregulation of IL-21R expression replace CD4⁺ T cell help of CD8⁺ T cell expansion *in vitro*. (A) IL-21R expression on CD8⁺ T cells stimulated with aAPC/mOKT3 in the presence or absence of CD4⁺ T cells was studied by flow cytometry. On the left, histogram plots for 1 donor is shown and, on the right, IL-21R expression on day 4 is displayed for 5 donors. (B) IL-21R expression on CD8⁺ T cells ectopically transduced with mock or IL-21R is shown (left). Expansion of transduced CD8⁺ T cells stimulated twice by aAPC/mOKT3 with or without IL-21 is compared (right). Percent expansion was calculated by dividing the number of expanded transduced CD8⁺ T cells by that of CD8⁺ T cells stimulated in the presence of CD4⁺ T cells. Values indicate mean of four independent experiments; error bars show s.d. * $P < 0.005$. doi:10.1371/journal.pone.0030229.g006

IL-2 and IL-21 have previously been shown to mediate CD4⁺ T cell help in murine *in vivo* studies. IL-2, one of the few effector cytokines made by naïve CD4⁺ T cells, expands activated T cells and is essential in the development of CD8⁺ T cell memory responses to pathogens [60]. While CD8⁺ T cell responses during acute viral infections were relatively independent of IL-2, the development of protective CD8⁺ T cell memory responses required IL-2 exposure during priming [35–37]. *In vivo* models also indicate that IL-21 is critical for containing chronic viral infections and preventing the deletion of high affinity antiviral CD8⁺ T cells. IL-21 secretion by CD4⁺ T cells enables the generation, sustained proliferation, and maintenance of polyfunctional CD8⁺ T cells during chronic infection [39–41].

Our results confirmed a role for IL-2 and IL-21 in human CD4⁺ T cell help. By using a standardized aAPC, we were able to single out and examine the effects of cocultured CD4⁺ T cells, unhindered by immunostimulatory and inhibitory factors produced by allogeneic feeder cells. Stimulation of T cells with aAPC/mOIKT3 induced the secretion of cytokines and chemokines, including high levels of interferon- γ , MIP-1 α , and MIP-1 β . Among all the cytokines and chemokines studied, blocking experiments identified IL-2 and IL-21 as necessary for CD4⁺ T cell help of CD8⁺ T cell expansion. These cytokines alone, however, were not sufficient to replace CD4⁺ T cells. We showed that CD4⁺ T cells help by enhancing IL-21R expression on CD8⁺ T cells, rendering them more responsive to secreted IL-21. Taken together, the secretion of IL-2/IL-21 and the induction of IL-21R are necessary and sufficient to partially recapitulate human CD4⁺ T cell help of CD8⁺ T cell expansion *in vitro*.

Transwell assays showed that the CD4⁺ T cell dependent expansion of CD8⁺ T cells was also mediated by cell-cell contact factors. CD40-CD40 ligand interactions have been shown to mediate CD4⁺ T cell help through CD40-mediated activation of dendritic cells, which are then “licensed” to stimulate CD8⁺ T cells [43,44,61]. CD40 ligation was also shown to increase IL-21R expression on B lymphocytes suggesting a mechanism for IL-21R upregulation on CD8⁺ T cells [62]. However, we did not observe any suppression of CD8⁺ T cell expansion following blockade of CD40 ligand (Figure 5D) even though expanded CD4⁺ T cells strongly expressed CD40 ligand (Figure 2C). Furthermore, stimulation with aAPC/mOIKT3 in the presence of CD40 ligation and the addition of IL-21 did not consistently enhance CD8⁺ T cell expansion (data not shown). Therefore, these results are in agreement with others who have shown that CD4⁺ T cells do not provide direct help to CD8⁺ T cells through CD40 ligation [63,64]. It should be noted that blocking of CD70, 4-1BB, or OX40 signaling also did not suppress the expansion of CD8⁺ T cells in the presence of CD4⁺ T cells (Figure 5D).

aAPC induced polyclonal expansion of both CD4⁺ and CD8⁺ T cells as shown by the absence of clonal skewing of the TCR V β repertoire. The ability to further expand antigen-specific T cells capable of killing tumor targets indicated that the TCR repertoire for highly avid T cells was preserved. Also, expanded TIL secreted higher amounts of Th1 cytokines, IFN- γ and IL-2, which are associated with anti-tumor immunity. While aAPC/mOIKT3 induced substantial expansion of CD8⁺ T cells in the presence of CD4⁺ T cell help, terminal effector T cell differentiation did not occur, as demonstrated by the central memory~effector memory phenotype (CD45RA⁻ CD45RO⁺ CD62L^{+/+}). Retention of CD62L expression would enable homing to lymph nodes, where encounter with antigen presented by professional APC could augment immune responses [65]. CD27, which is down-regulated in late stage effector T cells, was also highly expressed. CD27 expression by *in vitro* expanded TIL and T cell clones has been

associated with persistence and clinical responses after adoptive transfer [56,57,59,66].

We also found that expanded T cells were not contaminated by cells with the CD4⁺ CD25⁺ Foxp3⁺ Treg phenotype even when CD4⁺ CD25⁺ Foxp3⁺ T cells were present prior to stimulation. We previously found that K562-based aAPC expressing HLA-DR molecules did not expand Foxp3⁺ cells even though aAPC itself produces modest amounts of the Treg cell growth factor TGF- β [48]. We previously reported that aAPC also secretes IL-6 [47]. It is possible that IL-6, secreted by aAPC, might interfere with Foxp3⁺ Treg cell expansion [67,68].

Adoptive transfer of *in vitro* expanded T cells has led to clinically significant anti-tumor responses in patients [30]. By leveraging autologous CD4⁺ T cell help, aAPC/mOIKT3 eliminates the use of allogeneic feeder cells for T cell expansion, potentially increasing the availability of adoptive therapy as a cancer treatment. We previously reported the development of K562-based aAPCs dedicated to the expansion of HLA-restricted antigen-specific CD4⁺ and CD8⁺ T cells [47,48]. Antigen-specific CD4⁺ and CD8⁺ T cells expanded *in vitro* with these aAPC had a central memory~effector memory phenotype (CD45RA⁻ CD62L^{+/+}) and possessed surprisingly prolonged *in vitro* longevity without feeder cells or cloning. In a recent clinical trial, HLA-A2-restricted MART1 peptide-specific CD8⁺ T cells generated *in vitro* with aAPC were infused to advanced melanoma patients [69]. Without lymphodepletion or IL-2 administration, transferred T cells could persist for >16 months, established anti-tumor immunological memory *in vivo*, trafficked to tumor, and induced clinical responses. aAPC/mOIKT3 extends the K562 platform to the stimulation of T cells regardless of HLA subtype. The aAPC/mOIKT3-based T cell expansion system facilitates the understanding of mechanisms for human CD4⁺ T cell help and provides a novel strategy to expand T cells for *in vitro* and *in vivo* uses.

Materials and Methods

Ethics Statement

All specimens and clinical data were collected under protocols approved by the Institutional Review Board at the Dana-Farber Cancer Institute (DFCI). All patients provided written informed consent for the collection of samples and subsequent analysis.

CDNAs and cell lines

cDNAs encoding the heavy and light chains for a membranous form of anti-CD3 mAb (OKT3, mIgG2a) were cloned from hybridoma cells (ATCC, VA). HLA null K562 transduced with CD80 and CD83 has been described previously [47,53]. CD80⁺ CD83⁺ K562 cells were retrovirally transduced with the heavy and light chains of a membranous form of anti-CD3 mAb. After drug selection, anti-CD3 mAb expressing cells were isolated by magnetic bead guided sorting (Miltenyi Biotec, CA). High expression of a membranous form of anti-CD3 mAb on the cell surface was confirmed by flow cytometry. The parental cell line K562 lacks the endogenous expression of any HLA molecule, but does endogenously express the adhesion molecules CD54 and CD58.

Retrovirus supernatants expressing IL-21R was harvested from PG13 cells. Fresh CD8⁺ T cells purified from healthy donors were first activated with anti-CD3 (0.75 μ g/ml) and anti-CD28 (1 μ g/ml) mAbs (Fitzgerald Industries International, MA) for two days. Pre-activated T cells were infected with IL-21R or mock retrovirus supernatants every 24 hr at an MOI of 10 for 10 days and treated with 50 IU/ml IL-2 between infections. Following the assessment

of IL-21R expression by flow cytometry analysis, infected T cells were stimulated with aAPC/mOKT3.

T2, A375, and Malme-3M cell lines were obtained from ATCC as described elsewhere [47].

T cell expansion

Healthy donor PBMC were obtained by leukapheresis performed at the DFCI Kraft Family Blood Donor Center. Cells were isolated by Ficoll-Hypaque density gradient centrifugation and CD3⁺, CD4⁺, or CD8⁺ T cells were purified by negative selection via MACS sorting according to the manufacturer's protocol (Miltenyi Biotec, CA). TIL samples were processed by centrifugation of malignant ascites or mechanical and enzymatic digestion of melanoma metastases with collagenase as previously described [70]. CD3⁺ TIL were obtained by positive or negative selection via MACS sorting (Miltenyi Biotec, CA). aAPC/mOKT3 cells were irradiated (200 Gy) and added to purified T cells at a T cell to aAPC ratio of 20:1 unless otherwise noted. Dynabeads CD3/CD28 (Invitrogen, CA) were used as stimulators according to the manufacturer's instruction at a T cell to bead ratio of 1:3. Expanding T cells were cultured in RPMI 1640 containing 10% human AB sera and gentamycin (Invitrogen, CA), and between stimulations, unless otherwise noted, 300 IU/ml IL-2 (Prometheus, CA) was added every 3-4 days. In the absence of CD4⁺ T cells, CD8⁺ T cells expanded only in the presence of IL-2. Where indicated, 50 ng/ml IL-21 (Peptotech, NJ) was added every 3-4 days. Unless otherwise noted, T cells were restimulated every two weeks. Expanded cells were characterized two weeks after the second stimulation. Cell viability was >90% by trypan blue exclusion.

To test whether antigen-specific cultures can be generated from CD3⁺ T cells polyclonally expanded with aAPC/mOKT3, CD3⁺ T cells derived from HLA-A*0201 (A2)⁺ donors were initially stimulated and expanded with aAPC/mOKT3 for one month. Subsequently, CD8⁺ T cells were purified and further stimulated with Flu or MART1 peptide-pulsed aAPC/A2 as previously described [47,53].

Analysis of cultured T cells

Flow cytometry analysis was performed using mAbs for the following antigens: CD4, CD8, CD25, CD28, CD56, CD62L, and IL-2Rβ (Coulter, CA); CD40 ligand, CD80, IL-7Rα, OX40, OX40 ligand, and 4-1BB (BD Biosciences, CA); CD27, CD45RA, CD45RO and CD83 (Invitrogen, CA); CCR4 and CCR7 (R&D Systems, MN); ICOS, NKG2D, and PD-1 (eBioscience, CA); CD38, Foxp3, HLA-DR, and 4-1BB ligand (Biolegend, CA); CD40 and CD70 (Ancell, MN); IL-21R (R&D Systems, MN); or BD Biosciences, CA). Goat anti-mouse IgG (H+L) Fab (Jackson ImmunoResearch, PA) was used to detect surface expression of murine Ig. Assessment of TCR Vβ subfamily usage was performed using TCR Vβ mAbs (Beta Mark, Coulter, CA).

To assess the production/consumption of soluble factors in T cell cultures, purified CD4⁺, CD8⁺, or a 1:1 mixture of CD4⁺ and CD8⁺ T cells were stimulated with irradiated aAPC/mOKT3 for 72 hours and supernatants were measured for: GM-CSF, IFN-γ, IL-2, IL-4, IL-10, IL-12, IL-15, IL-17, MIP-1α, MIP-1β, RANTES, TNF-α, TNF-β, and TRAIL (R&D Systems, MN); IL-7 (Diaclone/Cell Sciences, MA); IL-18 (Medical & Biological Laboratories, Japan); and IFN-α (PBL Biomedical Laboratories, NJ). IL-21 (eBiosciences, CA) was measured at 48-hours. Relative changes in cytokines resulting from mixed cultures of CD4⁺ and CD8⁺ T cells vs. separate CD4⁺ and CD8⁺ T cell cultures were determined by the following formula: (x-y)/y, where x = cytokine secreted by CD4⁺ and CD8⁺ T cell mixed co-cultures and y is the

average of cytokine produced in separately stimulated CD4⁺ and CD8⁺ T cell cultures.

IFN-γ ELISPOT and standard chromium release assays were performed as described elsewhere [47,53]. IL-2, IL-4 and IL-10 ELISPOT assays were performed according to the manufacturer's protocol (R&D Systems, MN).

Transwell and blocking assays

Transwell assays were performed by placing purified CD4⁺, CD8⁺, or a mixture of CD4⁺ and CD8⁺ T cells into Millicell-24 plate chambers (Millipore) which were separated by a 0.4 μm filter allowing free movement of soluble factors but not cells. T cells were stimulated once with aAPC/mOKT3 in the absence of exogenous cytokines. Six days later, expansion of CD8⁺ T cells was determined.

Blocking assays were performed in 96-well round bottomed plates where CD4⁺ and CD8⁺ T cells were combined 1:1 and then stimulated with irradiated mOKT3/aAPC in the presence of blocking reagents. Blocking mAbs used recognized IL-2, IL-10, IL-15, IL-17R, MIP-1α, MIP-1β, OX40 ligand, RANTES, TNFα, and TNFβ (R&D Systems, MN); 4-1BB (Neomarkers, CA); CD40 ligand (Biolegend, CA); and CD70 (Ancell, MN). IL-21 was blocked using recombinant human IL-21R subunit/Fc chimeric protein (R&D Systems, MN) as previously described [71]. Six days later, CD8⁺ T cell expansion was determined.

Statistical analysis

Data analysis was performed using the paired, one-sided Student's t-test where $P < 0.05$ was considered to be statistically significant.

Supporting Information

Figure S1 K562-based aAPC/mOKT3, expressing a membranous form of anti-CD3 mAb, stimulates CD3⁺ T cell expansion. (A) CD3⁺ T cells were stimulated twice with aAPC/mOKT3 and supplemented with IL-2 at the following concentrations: 10 IU/ml (gray), 300 IU/ml (white) and 6,000 IU/ml (black). Fold expansion over 28 days is demonstrated. Without IL-2 addition, T cell expansion over the 28-day culture period was minimal. Data for three separate donors is shown. (B) CD3⁺ T cells were stimulated twice with aAPC/mOKT3 at the indicated aAPC: T cell ratios. Cultures were supplemented with IL-2 (300 IU/ml) between stimulations. Fold expansion of CD3⁺ T cells over one month is shown for two donors. (C) Phenotype of fresh healthy donor CD3⁺ T cells prior to stimulation is depicted to compare with the T cells shown in Figure 2C which were expanded with aAPC/mOKT3. Expression of surface molecules on gated CD4⁺ and CD8⁺ T cells is shown (open). Isotype mAb staining was used as a control (shaded). (D) HLA-A2⁺ healthy donor CD8⁺ T cells were stimulated with MART1 peptide-pulsed aAPC/A2 as previously described [47,53]. MART1 specific T cells were then stimulated twice with aAPC/mOKT3 in the presence of autologous CD4⁺ T cells. Fold expansion of MART1 T cells over one month is shown for three donors. (TIF)

Figure S2 TIL expanded with aAPC/mOKT3 express CD27 and CD28 and have a central memory~effector memory phenotype. CD3⁺ T cells from malignant ovarian ascites were stimulated twice with aAPC/mOKT3, and cultures were supplemented with IL-2 at 300 IU/ml. (A) Fresh, unstimulated TIL and (B) aAPC/mOKT3 expanded TIL were stained with indicated mAb (open) and isotype control (shaded).

TIL were analyzed after a one month expansion. Data depicted is on gated CD4⁺ and CD8⁺ T cells. (TIF)

Table S1 Soluble factors in T cell cultures stimulated with aAPC/mOKT3. Concentrations of soluble factors (pg/ml) in supernatants of CD4⁺ separate, or CD8⁺ separate, and CD4⁺ and CD8⁺ mixed T cell cultures stimulated by aAPC/mOKT3 were measured by ELISA. ^aPercent change was calculated as

detailed in Methods. ^bnot applicable. Data from two different donors is depicted. (DOC)

Author Contributions

Conceived and designed the experiments: MOB LMN NH. Performed the experiments: MOB OI MT SA AB GM MIM MMM APM NH. Analyzed the data: MOB LMN NH. Contributed reagents/materials/analysis tools: MOB OI YY MT SA HM LMN NH. Wrote the paper: MOB LMN NH.

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Mutations in the nucleolar phosphoprotein, nucleophosmin, promote the expression of the oncogenic transcription factor MEF/ELF4 in leukemia cells and potentiates transformation

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*Running title: *NPM1 mutations enhance HDM2 expression through MEF/ELF4*

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Background: MEF/ELF4 can function as an oncogene. We demonstrated the role of MEF/ELF4 in acute myeloid leukemia.

Results: NPM1 inhibited the DNA-binding and transcriptional activity of MEF/ELF4 on the HDM2 promoter whereas NPM1 mutant protein enhanced these activities of MEF/ELF4.

Conclusion: MEF/ELF4 activity may be activated by NPM1 mutant protein.

Significance: NPM1 mutant proteins have a role in MEF/ELF4-dependent leukemogenesis.

SUMMARY

Myeloid ELF1-like factor (MEF/ELF4), a member of the ETS transcription factors, can function as an oncogene in murine cancer models and is overexpressed in various human cancers. Here, we report a mechanism by which MEF/ELF4 may be activated by a common leukemia-associated mutation in the nucleophosmin gene. By using a tandem affinity purification assay, we found that MEF/ELF4 interacts with multifactorial protein nucleophosmin (NPM1). Coimmunoprecipitation and GST

pull-down experiments demonstrated that MEF/ELF4 directly forms a complex with NPM1 and also identified the region of NPM1 that is responsible for this interaction. Functional analyses showed that wild-type NPM1 inhibited the DNA-binding and transcriptional activity of MEF/ELF4 on the HDM2 promoter whereas NPM1 mutant protein (Mt-NPM1) enhanced these activities of MEF/ELF4. Induction of Mt-NPM1 into MEF/ELF4-overexpressing NIH3T3 cells facilitated malignant transformation. In addition, clinical leukemia samples with NPM1 mutations had higher human MDM2 (HDM2) mRNA expression. Our data suggest that enhanced HDM2 expression induced by mutant NPM1 may have a role in MEF/ELF4-dependent leukemogenesis.

Introduction

Myeloid ELF1-like factor (MEF/ELF4), a member of the ETS family of transcription factors, is characterized by an 85-amino acid ETS domain that recognizes a core sequence of GGAA or TTCC (1). MEF/ELF4 is

expressed in various normal and malignant hematopoietic cells and regulates the expression of various cytokines (interleukin-3 (1), granulocyte-macrophage colony stimulating factor (1), and interleukin-8 (2) as well as the cytolytic perforin molecule (3), antibacterial peptides lysozyme and human beta-defensin2 (4)), and matrix metalloproteinase (MMP)-9 expression (5). Furthermore, analyses of MEF/ELF4-deficient mice have revealed the essential role of MEF/ELF4 in the development and function of natural killer (NK) cells and NK-T cells (3). Recently, Smith *et al.* have shown that repression of Elf-4 by transcriptional repressor *Gfilb* is important for the maturation of primary fetal liver erythroid cells (6). MEF/ELF4 also regulates the key aspects of hematopoietic stem cell behavior by controlling movement through the cell cycle from quiescence (G0) to G1 and G1 to S as well as resistance to myelosuppression (7, 8).

MEF/ELF4 is expressed in cancers such as leukemia (9), lymphoma, and ovarian cancer (10). Recently, Totoki *et al.* identified an intra-chromosomal inversion (Xq25) in hepatocellular carcinoma that generated a BCORL1-MEF/ELF4 fusion transcript (11). Experiments in several mouse models have suggested that MEF/ELF4 plays a role in tumorigenesis. For example, models of retrovirus-induced insertional mutagenesis

have identified MEF/ELF4 as a gene that is involved in leukemic transformation (12). Sashida *et al.* have shown that overexpression of MEF/ELF4 enhances the expression of Mdm2, leading to decreased p53 expression (13) and enhanced transformation. In experiments with MEF/ELF4-overexpressing cells, they demonstrated that Ets1-induced p16 induction is suppressed, resulting in senescence suppression and tumor promotion.

Nucleophosmin (NPM1) is a nucleolar phosphoprotein (14) and a frequent target of genetic alterations in hematopoietic malignancies. NPM1 gene mutations have been found in approximately 60% of adult patients who have acute myeloid leukemia (AML) and a normal karyotype (15). These mutations lead to the aberrant cytoplasmic expression of NPM1 (NPMc+) due to nucleotide gain at the C-terminus (16, 17), which results in the loss of tryptophans residues essential for nucleolar localization and the gain of a new nuclear export signal (18). Increased NPM1 export into the cytoplasm probably perturbs multiple cellular pathways by delocalizing the proteins that interact with NPM1. By using a transgenic mouse model expressing the human NPMc+ mutation, it has been shown that NPMc+ confers a proliferative advantage in the myeloid lineage, suggesting that NPM1 mutations can participate in leukemia

development (19).

In the present study, we found that wild-type NPM1 (Wt-NPM1) downregulates, whereas mutated NPM1 (Mt-NPM1) upregulates, the transcriptional activity of MEF/ELF4 on the human MDM2 (HDM2) promoter. The expression of Mt-NPM1 in MEF/ELF4-overexpressing NIH3T3 cells resulted in enhanced malignant transformation. We also found that HDM2 mRNA expression in primary AML cells with *NPM1* mutations is significantly higher compared with AML cells without *NPM1* mutations. Taken together, our data suggest that NPM1 mutations may promote transformation by enhancing the oncogenic functions of MEF/ELF4.

EXPERIMENTAL PROCEDURES

Cell culture

293T cells (CRL-11268, ATCC, Manassas, VA, USA) were maintained at 37°C in DMEM (Invitrogen, Carlsbad, CA, USA) with bovine calf serum. U937 cells (CRL-1593.2; ATCC) were maintained with 10% (v/v) FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin (Fisher Scientific, Pittsburgh, PA, USA). NIH3T3 cells (CRL-1658; ATCC) were maintained under identical conditions with 10% (v/v) FBS and grown in RPMI 1640 (Fisher Scientific) with 10% FCS (HyClone, Logan, UT, USA), 100

U/mL penicillin G, and 100 µg/mL streptomycin. COS7 cells (CRL-1651; ATCC) were cultured in DMEM (Invitrogen) containing 10% FCS.

Tandem affinity purification assay

The cDNA of MEF/ELF4 was inserted into InterPlay N-terminal Mammalian TAP Vector (pTAP/MEF/ELF4; Stratagene, San Diego, CA, USA) comprising two affinity tags [immunoglobulin G (IgG)-binding domain and calmodulin-binding peptide] separated by the cleavage site of tobacco etch virus protease (20). 293T cells were transfected with pTAP or pTAP/MEF/ELF4 plasmids in a 10-cm dish. Transfected cells were collected and lysed in a solution containing 100 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 0.1% NP-40. The lysate was centrifuged at 15,000 rpm for 30 min at 4°C. The resulting supernatant was incubated for 2 h at 4°C with IgG-Sepharose 6 Fast Flow (GE Healthcare, Buckinghamshire, UK), after which the resin was washed and incubated with tobacco etch virus protease for 2 h at 16°C. Purification on calmodulin affinity resin (Stratagene) was performed according to the manufacturer's instructions. Purified proteins were precipitated with trichloroacetic acid, resolved with 1× sample buffer, and subjected to SDS-PAGE. Gels were stained with Coomassie blue, and protein bands were cut out. Proteins were