Cancer Immunotherapy Based on Mutation-Specific CD4+ T Cells in a Patient with Epithelial Cancer

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Limited evidence exists that humans mount a mutation-specific T cell response to epithelial cancers. We used a whole-exomic-sequencing-based approach to demonstrate that tumor-infiltrating lymphocytes (TIL) from a patient with metastatic cholangiocarcinoma contained CD4+ T helper 1 (Th1) cells recognizing a mutation in erbb2 interacting protein (ERBB2IP) expressed by the cancer. After adoptive transfer of TIL containing about 25% mutation-specific polyfunctional Th1 cells, the patient achieved a decrease in target lesions with prolonged stabilization of disease. Upon disease progression, the patient was retreated with a >95% pure population of mutation-reactive Th1 cells and again experienced tumor regression. These results provide evidence that a CD4+ T cell response against a mutated antigen can be harnessed to mediate regression of a metastatic epithelial cancer.

The human immune system has evolved to recognize and eliminate cells expressing foreign, nonself antigens. All malignant tumors harbor nonsynonymous mutations or other genetic alterations (1), some of which may generate neo-"nonself" epitopes that could potentially trigger an antitumor T cell response. Indeed, mutation-reactive T cells can frequently be found infiltrating human melanomas (2) and likely play a critical role in the clinical efficacy of adoptive cell therapy (ACT) and other immunotherapies in melanoma (3–7).

However, limited evidence exists demonstrating that the human immune system can mount an endogenous, mutation-specific T cell response against epithelial cancers that comprise over 80% of all human malignancies (8–11), and it is unclear whether this response can be harnessed to develop effective personalized cancer immunotherapies (12). Moreover, epithelial cancers often contain fewer mutations than melanoma (1), which may decrease the probability of eliciting a mutation-specific T cell response. We thus first set out to determine whether tumor-infiltrating lymphocytes (TIL) recognizing patient-specific mutations can be identified in patients with metastatic gastrointestinal (GI) cancers.

To this end, a 43-year-old woman with widely metastatic cholangiocarcinoma (patient 3737, table S1) who progressed through multiple chemotherapy regimens was enrolled in a TIL-based ACT protocol for patients with GI cancers (NCT01174121) (13). Lung metastases were resected and used as a source for whole-exomic sequencing and generation of T cells for treatment. Whole-exomic sequencing revealed 26 nonsynonymous mutations (table S2). To test whether the patient’s TIL recognized any of these mutations, we used a minigene approach. Briefly, for each mutation we designed a minigene construct that encoded for the mutated amino acid flanked on both sides of the mutation we designed a minigene construct that encoded for the mutated amino acid flanked on both sides of the mutant protein (fig. S1). Multiple minigenes were synthesized in tandem to generate tandem transgenic lines (140–141, 180, 233, 342, 6001, 2322–2335, 2010). The results provide evidence that a CD4+ T cell response against a mutated antigen can be harnessed to mediate regression of a metastatic epithelial cancer.

References and Notes
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Supplementary Materials
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Materials and Methods
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individually transfected into autologous antigen-presenting cells, followed by a coculture with TIL to determine whether any of the processed and presented mutated antigens were recognized by TIL. We observed reactivity of 3737-TIL to a mutated antigen present in TMG-1 but not TMG-2 or TMG-3 (Fig. 1A). Moreover, the reactivity predominated in the CD4+ T cell population, as demonstrated by up-regulation of the activation markers OX40 and 4-1BB (Fig. 1B).

Although some 4-1BB up-regulation was observed in the CD4-negative (CD8+) T cell population, we sorted these cells and found no reactivity against the TMG. To determine which of the nine mutations in TMG-1 was being recognized by 3737-TIL, we synthesized nine additional TMG-1 constructs, each one containing a reversion of one of the mutations back to the wild-type sequence. Reactivity of 3737-TIL to TMG-1 was abrogated only when the erbb2 interacting protein (ERBB2IP) mutation was reverted back to the wild-type sequence, indicating that the TIL specifically recognized the ERBB2IP<sup>E805G</sup> mutation (Fig. 1C). The response was restricted by HLA-DQB1*0601, and the minimal epitope was located within the 13–amino acid sequence NSKEETGHLENGN (where E is Glu; G, Gly; H, His; K, Lys; L, Leu; N, Asn; S, Ser; and T, Thr) (fig. S2).

We next characterized the clonality of the mutated ERBB2IP-specific CD4+ T cells by sorting them after antigen-specific activation, using OX40 as a marker of activation (fig. S3A). These cells were then bulk-expanded and cloned by limiting dilution. A flow cytometry-based survey of the T cell receptor (TCR)-V<sub>b</sub> repertoire demonstrated that the bulk-expanded population was >95% V<sub>b</sub>22+ and that 10/11 clones assessed were purely V<sub>b</sub>22+ (fig. S3, B and C). TCR sequence analysis revealed the same TCR<sub>b</sub> variable, diversity, joining (V-D-J) sequence in 6/6 V<sub>b</sub>22+ clones tested (table S4), suggesting that the majority of the ERBB2IP<sup>E805G</sup> mutation–reactive T cells was composed of a dominant V<sub>b</sub>22+ T cell clone. T cell clones expressing this V<sub>b</sub>22 TCR specifically produced the cytokine interferon (IFN)-γ upon stimulation with the mutated ERBB2IP peptide (Fig. 1D). Moreover, autologous open-repertoire peripheral blood T cells were transduced (Td) with nothing (Mock) or the TCR derived from the V<sub>b</sub>22+ clone and then assessed for reactivity as described in (D). The endogenous V<sub>b</sub>22+ TCR constant regions were swapped with mouse constant regions, allowing for the detection of the introduced TCR using antibodies against the mouse TCR<sub>b</sub> constant region (mTCR<sub>b</sub>). Plate-bound OKT3 was used as a control in all assays. All data are representative of at least two independent experiments. Error bars are SD.

The patient underwent adoptive transfer of 42.4 billion TIL containing CD4+ ERBB2IP mutation–reactive T cells followed by four doses of interleukin (IL)-2 to enhance T cell proliferation and function (fig. S4). Flow cytometry analysis demonstrated that about 25% of the entire 3737-TIL product administered was composed of the

**Fig. 1.** Patient 3737–TIL harbor ERBB2IP mutation–specific CD4+ T cells. (A to C) Patient 3737–TIL were cocultured with dendritic cells (DCs) transfected with nothing (Mock), green fluorescent protein (GFP) RNA, or the indicated TMG construct encoding the various mutations identified by whole-exomic sequencing. (A) IFN-γ enzyme-linked immunosorbent assay at 20 hours. “>” denotes greater than 500 spots per 1e3 cells. (B and C) Flow-cytometric analysis at 24 hours. Data are gated on live CD3+ cells and, for (C), further gated on CD4+ cells. (D) A CD4+ V<sub>b</sub>22+ clone was co-cultured for 6 hours with autologous B cells pulsed overnight with wild-type (wt) ERBB2IP, mutated (mut) anaplastic lymphoma receptor tyrosine kinase (ALK), or mut ERBB2IP 25–amino acid–long peptides. Flow cytometry was used to detect intracellular IFN-γ production in the CD4+ population. Data are representative of two clones sharing the same TCR-V<sub>b</sub> sequence. (E) Autologous open-repertoire peripheral blood T cells were transduced (Td) with nothing (Mock) or the TCR derived from the V<sub>b</sub>22+ clone and then assessed for reactivity as described in (D). The endogenous V<sub>b</sub>22+ TCR constant regions were swapped with mouse constant regions, allowing for the detection of the introduced TCR using antibodies against the mouse TCR<sub>b</sub> constant region (mTCR<sub>b</sub>). Plate-bound OKT3 was used as a control in all assays. All data are representative of at least two independent experiments. Error bars are SD.
Vb22+, mutation-reactive T cells (Fig. 2, A and B), equating to the infusion of over 10 billion ERBB2IP mutation–specific CD4+ T cells. Elevated levels of IFN-γ were detected in the patient’s serum for the first 5 days after cell infusion (Fig. 2C). Although the patient had clear evidence of progressive disease before the cell infusion, tumor regression was observed by the 2-month follow-up, and all target lung and liver lesions continued to regress, reaching a maximum reduction of 30% at 7 months post-treatment (Fig. 2D). The patient experienced disease stabilization for about 13 months after cell infusion, after which disease progression was observed only in the lungs but not liver.

To determine whether there was evidence that the CD4+ ERBB2IP mutation–reactive T cells played a role in the disease stabilization, we evaluated the in vitro phenotype and function and in vivo persistence of these cells. A number of preclinical studies (14–20) and one case report in melanoma (21) have demonstrated that T helper 1 (TH1) cells, through a variety of mechanisms including the secretion of the pleiotropic cytokines IFN-γ and tumor necrosis factor (TNF) (22, 23), can mediate tumor regression. Moreover, CD4+ T cells that can simultaneously produce multiple effector cytokines (polyfunctional T cells) have been correlated with effective antitumor T cell responses (19, 24). We found that the Vβ22+ ERBB2IP mutation–reactive CD4+ T cells were polyfunctional T effector cells, because stimulation with the mutated ERBB2IP peptide induced the robust coexpression of IFN-γ, TNF, and IL-2 (Fig. 3A) but little to no IL-4 or IL-17. Further phenotypic characterization revealed that these cells were predominantly effector memory CD4+ T cells with cytolytic potential (Fig. S5, A and B).
There also appeared to be a minor population of polyfunctional Vß22-negative, ERBB2IP mutation–reactive CD4+ T cells present in 3737-TIL (Figs. 2B and 3A). Sorting of the Vß22-negative cells followed by activation of these cells revealed that one or more additional clonotypes reactive to this epitope were present in 3737-TIL (fig. S6, A and B), the most dominant clonotype of which was Vß5.2 (fig. S6, C and D). The majority of the Vß5.2+ cells produced multiple cytokines in an antigen-specific manner (fig. S6E and table S6). There also appeared to be a minor population of Vß5.2-negative (and Vß22-negative) CD4+ T cells that recognized mutated ERBB2IP (fig. S6E). Thus, the TIL used to treat patient 3737 contained at least three different polyfunctional CD4+ T cell clones that recognized the same mutation in ERBB2IP, suggesting that this mutation was highly immunogenic.

The in vivo persistence of adoptively transferred T cells in the blood at 1 month post–cell transfer is correlated with improved outcomes in melanoma patients treated with ACT (25). We thus evaluated the in vivo persistence of the ERBB2IP mutation–reactive CD4+ T cell clones in patient 3737. TCR-Vß deep sequencing revealed that these clonotypes were rare or not detectable in the peripheral blood before ACT (Fig. 3B). Ten days after ACT, both clones were present at greater than 2% of the total T cells in the peripheral blood, but declined to less than 0.3% by day 34 post–cell infusion (Fig. 3B). Three lung metastases, which were resected nearly a year and a half after ACT, were infiltrated by the ERBB2IP mutation–reactive T cells (Fig. 3B), suggesting that these cells contributed to the cancer regression and disease stabilization. The Vß22+ ERBB2IP mutation–reactive clone was the most frequent clone detected in tumor nodule–3 (Tu-3-Post) and represented nearly 8% of total T cells in the tumor (Fig. 3B), whereas this clone was the second and twelfth most frequent in tumor nodules–1 and –2, respectively. The Vß5.2+ ERBB2IP mutation–reactive clone was also enriched compared with its frequency in blood in all three tumor nodules (Fig. 3B). Thus, patient 3737 experienced tumor regression with stabilization of disease for more than 1 year after receiving over 10 billion ERBB2IP mutation–specific polyfunctional T cells that infiltrated and persisted in the metastatic lesions.

We observed relatively high levels of ERBB2IP expression in both the original and the recurrent lung lesions as determined by quantitative reverse transcription polymerase chain reaction (fig. S7A), and Sanger sequencing validated the presence of the ERBB2IP mutation in all tumor lesions (fig. S7B). The T cell infiltrate and major histocompatibility complex expression of the tumors in situ are summarized in tables S7 and S8, respectively. The presence of T cells reactive to the ERBB2IP mutation in progressing tumors that expressed the mutation and persisted over time suggests the presence of immunosuppressive effects at the tumor site that may need to be overcome to increase the anti–cancer effects of the transferred cells.

To specifically evaluate the contribution of mutation-reactive T cells to the antitumor response in vivo, we generated and adoptively transferred a TIL product that was comprised of >95% of the Vß22+ ERBB2IP mutation–reactive T cells. (Fig. 4). This approach was used to generate a T cell product that was highly enriched in mutation-reactive T cells for use in ACT. The ability to immunologically target unique mutations in cancers can potentially extend highly personalized immunotherapies to patients with epithelial cancers, which account for about 90% of cancer deaths in the United States.

Fig. 4. Evidence of tumor regression after treatment with a highly pure population of Vß22+ ERBB2IP mutation–reactive CD4+ T cells. (A) Flow-cytometric analysis of the TIL product used for the first infusion. Data are gated on live CD3+ T cells (left) and further gated on CD4+ cells (right). (B) Retreatment TIL were co-cultured for 6 hours with autologous B cells pulsed overnight with wt or mut ERBB2IP 25–amino acid–long peptides. Flow cytometry was used to detect intracellular TNF production in the CD4+ population. (A) and (B) are representative of at least two independent experiments. (C) Tumor growth curves (sum of maximum diameters) of patient 3737 before and after first and second adoptive cell transfusions. The first infusion product consisted of 24.2 billion T cells containing about 25% (10 billion) Vß22+ ERBB2IP mutation–reactive TIL cells. The second infusion product consisted of 126 billion T cells containing over 95% (120 billion) Vß22+ ERBB2IP mutation–reactive TIL cells. Data are expressed as a change from baseline and stratified on lung, liver, and total tumors. Some target lesions selected in (C) differ from Fig. 2D because only lesions that were present throughout both treatments were selected for measurement.

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The Transcription Factor Gata6 Links Tissue Macrophage Phenotype and Proliferative Renewal

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Tissue-resident macrophages are heterogeneous as a consequence of anatomical niche–specific functions. Many populations self-renew independently of bone marrow in the adult, but the molecular mechanisms of this are poorly understood. We determined a transcriptional profile for the major self-renewing population of peritoneal macrophages in mice. These cells specifically expressed the transcription factor Gata6. Selective deficiency of Gata6 in myeloid cells caused substantial alterations in the transcriptome of peritoneal macrophages. Gata6 deficiency also resulted in dysregulated peritoneal macrophage proliferative renewal during homeostasis and in response to inflammation, which was associated with delays in the resolution of inflammation. Our investigations reveal that the tissue macrophage phenotype is under discrete tissue-selective transcriptional control and that this is fundamentally linked to the regulation of their proliferation renewal.

Many resident macrophages self-renew by local proliferation [(1) and references therein]. This is initiated after seeding of macrophages into tissues during development and their expansion during the neonatal period ([1]). Under specific conditions, these tissue-resident macrophages may also be derived from blood monocytes ([1]). Classic F4/80highCD11bhigh peritoneal-resident macrophages fit this model ([2]–[7]), and they proliferate above homeostatic levels in response to inflammation ([6]). Proliferation of human macrophages has also been observed in several contexts [reviewed in (1)]. However, the factors controlling these processes remain ill-defined. We hypothesized that discrete transcriptional controls would govern both the specific phenotype of tissue

Fig. 1. Selective myeloid cell alterations in the peritoneum of mice with myeloid Gata6 deficiency. (A) Representative flow cytometric and immunofluorescent assessment of peritoneal-resident macrophages from WT and Gata6-KOmye mice. F4/80high (arrowhead) and F4/80low (arrows) macrophages are indicated. Fluorescent images were captured with a ×40 objective lens, the scale bar is indicated, and the images are representative of four mice per group (fig. S9). (B) Representative flow cytometric analysis of the peritoneal myeloid cell (CD11b+CD19+) composition of the Gata6-WT and Gata6-KOmye mice. Percentages indicate typical proportions of the cell types of all peritoneal cells. (C) Quantification of peritoneal myeloid cells in the Gata6-WT (black bars, n = 9/37[♀]) and Gata6-KOmye mice (white bars, n = 5/37[♀]) analyzed by flow cytometry in (A) and (B) above. Data represent the mean ± SEM of mice pooled from two independent experiments and were analyzed by two-way analysis of variance (ANOVA) MØ, macrophage; Res, tissue-resident; Eos, eosinophil; DC, dendritic cell; Int, interaction statistic; Gata6, Gata6 effects; Sex, sex effects.
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