A Mucin 16 bispecific T cell–engaging antibody for the treatment of ovarian cancer

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Advanced ovarian cancer is frequently treated with combination chemotherapy, but high recurrence rates show the need for therapies that can produce durable responses and extend overall survival. Bispecific antibodies that interact with tumor antigens on cancer cells and activating receptors on immune cells offer an innovative immunotherapy approach. Here, we describe a human bispecific antibody (REGN4018) that binds both Mucin 16 (MUC16), a glycoprotein that is highly expressed on ovarian cancer cells, and CD3, thus bridging MUC16-expressing cells with CD3+ T cells. REGN4018 induced T cell activation and killing of MUC16-expressing tumor cells in vitro. Binding and cytotoxicity of REGN4018 in vitro were minimally affected by high concentrations of CA-125, the shed form of MUC16, which is present in patients. In preclinical studies with human ovarian cancer cells and human T cells in immunodeficient mice, REGN4018 potently inhibited growth of intraperitoneal ovarian tumors. Moreover, in a genetically engineered immunocompetent mouse expressing human CD3 and human MUC16 [humanized target (HuT) mice], REGN4018 inhibited growth of murine tumors expressing human MUC16, and combination with an anti–PD-1 antibody enhanced this efficacy. Immuno-PET imaging demonstrated localization of REGN4018 in MUC16-expressing tumors and in T cell–rich organs such as the spleen and lymph nodes. Toxicology studies in cynomolgus monkeys showed minimal and transient increases in serum cytokines and C-reactive protein after REGN4018 administration, with no overt toxicity. Collectively, these data demonstrate potent antitumor activity and good tolerability of REGN4018, supporting clinical evaluation of REGN4018 in patients with MUC16-expressing advanced ovarian cancer.

INTRODUCTION

Ovarian cancer is the most lethal of the gynecologic malignancies (1, 2), partially because it is frequently diagnosed at an advanced stage (3). The current standard of care for ovarian cancer is surgery, followed by chemotherapy with a combination of platinum agents and taxanes (4). Although most patients respond to initial treatment, most experience a recurrence of the disease, resulting in a cycle of repeated surgeries and additional rounds of chemotherapy. Despite recent advances in therapy such as poly(adenosine 5′-diphosphate–ribose) polymerase inhibitors for patients carrying BRCA or other homologous recombination deficiency mutations, advanced ovarian cancer remains a disease of high unmet need (5–7).

Evidence suggests that ovarian cancer may be amenable to some forms of immunotherapy (8). For example, patients with ovarian cancer whose tumors were positive for intraepithelial CD8+ T lymphocyte infiltration had notably better overall and progression-free survival than patients without intraepithelial CD8+ T lymphocyte infiltration, suggesting that T cells can play a role in controlling tumor growth (9, 10). Moreover, some patients have shown evidence of immune response to their tumors, demonstrated by detection of tumor-reactive T cells and antibodies in the blood, tumor, or ascites of patients with advanced disease (11–14). Blockade of the programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) checkpoint pathway has shown some benefit in ovarian cancer; PD-1 blockade monotherapy resulted in an overall response rate of about 10 to 15% in early clinical trials (9). Thus, blockade of this pathway alone has shown only modest clinical responses.

CD3-engaging bispecific antibodies are emerging as a potent immunotherapy for the treatment of cancer because they can broadly activate CD3-expressing T cells in the presence of tumor cells. The only clinically approved CD3 bispecific molecule is blinatumomab, a CD19×CD3-targeting molecule for acute lymphoblastic B cell leukemia (15). Given that the bispecific therapeutic approach induces polyclonal T cell activation, they may be efficacious in a low neoantigen burden disease such as ovarian cancer.

Because of the potency of a bispecific antibody, it is essential to identify a tumor target antigen with sufficient tumor selectivity to limit toxicity. Mucin 16 (MUC16) is a large integral membrane glycoprotein that is highly expressed in a number of epithelial cancers (16) but at low abundance in epithelial cells of several tissues including trachea, eye, and female reproductive organs, as well as the serosal epithelial lining of the peritoneal and thoracic cavity. MUC16 is thought to provide a barrier function in some of these organs (17, 18). Proteolytic cleavage of cell surface MUC16 results in the shedding of the extracellular portion of MUC16 [known as cancer antigen 125 (CA-125)] into the bloodstream and a short, membrane-associated C-terminal domain that remains on the cell surface. Circulating CA-125 is a biomarker of ovarian cancer (17–19). Agents using several MUC16-targeted approaches are in early phases of clinical development, such as chimeric antigen receptor (CAR) T cells (20) and antibody-drug conjugates (21). MUC16 can be detected in...
around 80% of ovarian carcinomas and is expressed on all ovarian subtypes (serous, mucinous, endometrioid, and clear cell), although expression is heterogeneous (22, 23). Because of its up-regulation in tumors and apical expression on normal tissue where it may be less accessible to therapeutic antibodies, MUC16 is a viable and attractive ovarian tumor target.

Here, we describe the design, pharmacological activity, and nonclinical safety assessment of a T cell–dependent bispecific antibody targeting MUC16 on tumor cells. Collectively, the results provide the foundation for clinical testing of REGN4018 in patients with MUC16-expressing advanced ovarian cancer.

RESULTS
REGN4018 binds to MUC16-expressing tumor cells and CD3 on human and cynomolgus monkey T cells
CD3-binding bispecific antibodies that redirect T cells to kill MUC16-expressing ovarian cancer cells were generated using VelocImmune mice (24, 25), which express human immunoglobulin genes. REGN4018 resulted from an anti-MUC16 antibody and an anti-CD3 antibody, using the light chain from the MUC16 antibody (Fig. 1A). The constant region of REGN4018 is based on a hinge-stabilized, effector function–minimized version of the immunoglobulin G4 (IgG4) isotype as previously described (16, 26) and shown in Fig. 1A. Cell surface binding of REGN4018 to human and cynomolgus monkey T cells was assessed by flow cytometry using peripheral blood mononuclear cells (PBMCs). REGN4018 bound to both human and cynomolgus T cells. The binding to cynomolgus T cells was weaker than that to human T cells (Fig. 1B). To assess binding to MUC16, tumor cell lines expressing a range of MUC16 abundance were selected. The antibody binding capacity for REGN4018 on endogenously expressing cells, defined as the number of antibody molecules bound to the cell surface under saturating conditions, ranged from 26,000 on CFPAC-1 cells (pancreatic cancer cells) to 300,000 on OVCAR-3 cells (ovarian cancer cells), demonstrating that REGN4018 binds tumor cells expressing a range of MUC16 (Fig. 1C). REGN4018 specifically bound to all MUC16-expressing cell lines tested with half maximal effective concentration (EC₅₀) values ranging from 1.7 nM for the endogenously expressing CFPAC-1 cells to 2.15 nM for ID8 mouse ovarian tumor cells engineered to express a membrane-proximal portion of human MUC16 up to the fifth SEA (sea urchin sperm protein, enterokinase, and agrin) domain [ID8–vascular endothelial growth factor (VEGF)/huMUC16Δ] (denoted in Fig. 1D). The nonbinding control antibody did not bind to any tested cell lines (Fig. 1E), and REGN4018 did not bind the ID8-VEGF parental cells, which were included as a negative control cell line (Fig. 1E).

REGN4018 induces target-dependent T cell activation and cytotoxicity in the presence of CA-125
The ability of REGN4018 to activate T cells was investigated in a nuclear factor of activated T cells–Luciferase (NFAT-Luc) reporter bioassay using engineered cells expressing human or cynomolgus (cyno) CD3 (Jurkat NFAT-Luc or Jurkat NFAT-Luc/cynoCD3, respectively). REGN4018 activated human CD3-mediated NFAT signaling in the presence of human OVCAR-3 cells, as well as monkey CD3-mediated NFAT signaling in the presence of monkey MUC16-expressing ID8-VEGF cells (Fig. 2A). The cytotoxic potency of

Fig. 1. REGN4018 binds to human and monkey CD3 and MUC16. (A) Schematic of bispecific antibody format. Heavy-chain variable regions of different specificities are in red and green. Red asterisk indicates the Fc mutation used for purification. (B) REGN4018 binding to CD3 on human and cynomolgus monkey T cells (CD2/CD16) shown by flow cytometry (two replicates). (C) The number of MUC16 epitopes per cell on the cell lines is reported as antibody binding capacity. (D) Schematic of MUC16 including the “membrane-proximal region” where the antibody binds. (E) Binding of REGN4018 to human and cynomolgus monkey MUC16 on a range of tumor cell lines shown by flow cytometry. Isotype control, green; REGN4018, blue (two replicates).
REGN4018 was assessed in a flow cytometry-based cell killing assay. REGN4018 induced human T cells and cynomolgus T cells to kill OVCAR-3 cells (EC50, 1.36 × 10^{-11} M and 3.06 × 10^{-11} M, respectively) (Fig. 2B). OVCAR-3 cells were used in this assay because they express the large natural MUC16 glycoprotein, validating that REGN4018 can induce killing of MUC16-expressing cells in the presence of full-length MUC16. The cytotoxic activity of human and cynomolgus T cells to OVCAR-3 cells was accompanied by up-regulation of the T cell activation marker PD-1 (Fig. 2C). Comparison of CD69 and PD-1 up-regulation in response to anti-CD3/anti-CD28 stimulation confirms the ability of REGN4018 to potently activate T cells (performed twice). Significance measured by unpaired test in comparison to CD3-binding control (* P < 0.05, ** P < 0.01, and *** P < 0.001). (E) Human T cell killing of ID8-VEGF/huMUC16Δ (left), ID8-VEGF/cynoMUC16 (middle), or ID8-VEGF cells (right). (F) Flow cytometry measurements of REGN4018 (blue) or anti-MUC16 clone 3A5 (black) binding alone or in the presence of CA-125 or a truncated MUC16. (G) Killing of OVCAR-3 cells by human PBMCs in the presence of REGN4018 with increasing concentrations of CA-125 (left) or truncated MUC16 (right) (performed twice).

CA-125 is increased in the serum of most patients with ovarian cancer (27), and circulating CA-125 could affect any MUC16-targeted therapy by acting as an antigen sink. We therefore examined whether the functional activity of REGN4018 is affected by the presence of soluble CA-125. We performed flow cytometry to examine binding and cytotoxicity assays in the presence of high amounts of CA-125 purified from ascites of patients with ovarian cancer. The concentrations of CA-125 used in the assay (10,000 U/ml) greatly exceed the median published serum concentration of 656.6 U/ml found in patients with serous ovarian cancer (28). Excess CA-125 had minimal impact on REGN4018 binding to OVCAR-3 cells, suggesting minimal binding to CA-125. In contrast, CA-125 inhibited the ability of a comparator anti-MUC16 antibody by 70% (in-house version of antibody clone 3A5) (Fig. 2F). On the other hand, a soluble MUC16 construct containing the membrane-proximal region of MUC16 (MUC16Δ) substantially inhibited binding of REGN4018, demonstrating that REGN4018 binds this membrane-proximal region (Fig. 2F). Minimal binding to CA-125 was confirmed by enzyme-linked immunosorbent assay (fig. S3). The limited binding to the
REGN4018 mediates potent T cell–mediated killing of human ovarian cancer cells (OVCAR-3) growing intraperitoneally in mice

To assess the in vivo antitumor activity of REGN4018, we established a tumor xenograft ascites model using OVCAR-3 ovarian cancer cells. We generated OVCAR-3 cells stably expressing the firefly Luc reporter enzyme to monitor tumor growth using in vivo bioluminescence imaging (BLI). To evaluate redirected killing induced by REGN4018, human T cells were provided by transferring human PBMCs to mice before tumor cell implantation. OVCAR-3/Luc cells were then intraperitoneally implanted into mice, and treatment began on day 6 (Fig. 3A, left) or day 7 (Fig. 3A, right) after tumor implantation. Mice treated with REGN4018 (0.05 mg/kg or higher) once tumor growth was established had significantly reduced tumor burden compared to mice treated with either a nonbinding control antibody or a CD3-binding control antibody (Fig. 3, A and B). REGN4018 treatment did not result in any changes in weight (fig. S4, A and B). Changes in weight and body condition scoring were also used as a readout for graft-versus-host disease (GVHD), which is a common phenomenon in this xenogenic model. Experiments were completed before GVHD was evident. Circulating REGN4018 was detected in the serum at all assessed time points for the two higher dose groups (fig. S4C). Tumor cells remaining in the peritoneal cavity maintained MUC16 expression (fig. S5).

Upon treatment, BLI abundance similar to those in nontumor-bearing mice (baseline) should correspond to complete tumor clearance. This was confirmed using flow cytometry; in mice with BLI that had returned to baseline after therapy, no tumor cells were detected at the end of the study. In contrast, mice treated with the CD3-binding control bispecific maintained high BLI and contained OVCAR-3/Luc cells in the peritoneal cavity (fig. S6, A and B). To enable examination of tumor burden for a longer period of time, mice were depleted of T cells to avoid development of GVHD and followed for 57 days after treatment stopped. No tumor growth was detected, and mice did not gain weight because of asites in the REGN4018-treated group (fig. S6C). Elevated serum CA-125 can be detected in mice with high tumor burden in this model. CA-125 concentrations were significantly higher in control-treated mice compared to those treated with the two higher doses of REGN4018, and the amount of CA-125 in the serum correlated with tumor burden (BLI), showing that serum CA-125 can be used as a readout of therapeutic response (Fig. 3C).

To assess in vivo T cell activation by REGN4018 in mice bearing OVCAR-3 tumors and human PBMCs, serum cytokines from tumor-bearing mice were measured. Serum samples were collected 4 hours after the first antibody dose in the REGN4018 (0.5 mg/kg), CD3-binding control, and nonbinding control groups. Treatment with REGN4018 activated T cells as determined by induction of IFN-γ, TNFα (tumor necrosis factor–α), IL-2, IL-6, IL-8, and IL-10, compared to the nonbinding control and the CD3-binding control (Fig. 3D). REGN4018 does induce a substantial serum cytokine response, and the concentrations of cytokines are higher when the mice are administered anti-CD3, which induces a systemic T cell activation via Fc receptor cross-linking (fig. S7A). Cell types other than T cells are unlikely to contribute to the human cytokine production because only T cells are maintained in the NSG mice in any appreciable frequency, with a slight skewing toward CD8+ T cells over CD4+ T cells (fig. S7B). Antitumor efficacy required the presence of human T cells (fig. S8A), and REGN4018-induced cytokine response required the presence of both human T cells and OVCAR-3/Luc cells (fig. S8B). Examination of T cells in the peritoneal cavity 48 hours after dosing showed that REGN4018 induced expression of CD25, PD-1, and granzyme B—all markers of T cell activation (Fig. 3E). REGN4018 did not deplete T cells because T cells were still present in the spleen and ascites of mice treated with REGN4018 (Fig. 3F).

Mice expressing human MUC16 and CD3 have a normal T cell compartment, and REGN4018 induces no inflammation in MUC16-expressing tissues

To investigate the antitumor efficacy of REGN4018 in a mouse with a fully intact immune system, mice were genetically engineered to express both human CD3 on T cells and the membrane-proximal region of MUC16 in the endogenous murine loci (knockin mice) (Fig. 4A). To validate these mice, MUC16 expression was examined by both reverse transcription polymerase chain reaction and immunohistochemistry (IHC). mRNA expression was detected in the trachea and at low abundance in the lung, heart, ovary, pancreas, and bladder, similar to murine MUC16 expression (Fig. 4B) (29). To assess MUC16 protein expression, IHC was performed on OVCAR-3 cells and selected tissues using an antihuman MUC16 antibody that recognizes a membrane-proximal region of MUC16 (Fig. 4C). MUC16 protein expression was confirmed in the surface epithelium of the ovary and stomach, the tracheal lining epithelium, and the submucosal glands, as described in humans (Fig. 4C, right) (17, 29).

The T cells in these mice express human CD3, are polyclonal as assessed by T cell receptor (TCR) Vβ usage (Fig. 4D), and are present in similar numbers to wild-type mice (Fig. 4E). To determine whether REGN4018 induced any T cell activation or effects on normal tissues in these animals, nontumor-bearing mice were injected with a high dose of REGN4018 (10 mg/kg), and T cell numbers in blood, serum cytokines, and histopathology were examined. It has previously been described that, upon stimulation through CD3, a transient loss of T cells from the blood is observed. Although T cells can be activated by an antihuman CD3 antibody (OKT3) as measured by loss of T cells from the blood and increased serum cytokines, REGN4018 did not induce any such effects, suggesting limited accessibility of the MUC16 target (Fig. 4, F and G). To determine whether REGN4018 induced any microscopic changes in MUC16-expressing tissues, MUC16 and CD3 humanized target (HuT) mice received two doses of REGN4018 at 10 mg/kg on days 0 and 3. On day 5, several MUC16-expressing tissues (trachea, stomach, and ovary) were examined, and no cellular infiltration or necrosis was seen in these tissues (Fig. 4H).

In vivo immuno-PET imaging and biodistribution of REGN4018 show targeting to secondary lymphoid organs through CD3 binding and MUC16-driven targeting to the tumor

The in vivo localization of REGN4018 and the expression of MUC16 protein were assessed in wild-type and genetically HuT mice using...
positron emission tomography (PET) imaging. The biodistribution of the $^{89}$Zr-labeled anti-MUC16 parental antibody was similar in both wild-type and HuT mice, suggesting low expression/availability of the HuT MUC16 protein to the antibody. In contrast, when mice were administered therapeutically relevant doses of $^{89}$Zr-labeled REGN4018 (the bispecific antibody), distribution to the spleen (yellow arrow) and lymph nodes (LNs; green arrow) was evident, likely because of recognition of CD3+ T cells in these lymphoid organs (Fig. 5A). Ex vivo biodistribution analyses in individual tissues confirmed the localization to LNs and spleen (Fig. 5B). To assess whether REGN4018 can accumulate in MUC16-expressing tumors, $^{89}$Zr-labeled REGN4018 was administered to mice bearing ID8-VEGF-huMUC16Δ tumors. Although REGN4018 still accumulated in the spleen (yellow arrow) and LNs (green arrow) because of CD3 targeting, tumor distribution was also evident (red arrow), indicating that REGN4018 can localize to MUC16-expressing tumors in animals with an intact immune system and endogenous expression of CD3 and MUC16 (Fig. 5C). The addition of the MUC16 parental antibody to block MUC16 availability at the time of dosing with $^{89}$Zr-labeled REGN4018 reduced tumor targeting, demonstrating that at least some of the targeting to the tumor is MUC16 driven. The addition of a CD3-binding control antibody reduced targeting to spleen and LN to almost baseline, and targeting to the MUC16-expressing tumor was enhanced (Fig. 5, C and D).

REGN4018 is efficacious in two syngeneic tumor models in HuT mice

To investigate the antitumor efficacy of REGN4018 in an immunocompetent mouse model, we used the genetically engineered mice described above and the syngeneic tumor cell line ID8-VEGF/huMUC16Δ. All mice in these studies were given a total of five doses of REGN4018 or a CD3 binding control. REGN4018 significantly inhibited subcutaneously implanted ID8-VEGF/huMUC16Δ tumors compared to the CD3-binding control when treatment began on the day of tumor implantation or was delayed until day 10 after tumor implantation (Fig. 6A). In an ascites model, REGN4018 resulted in a markedly longer median survival time than control-treated mice.
Expression of huMUC16Δ was maintained (although slightly reduced) on the tumor cells at end of study as measured by flow cytometry (Fig. 6C). REGN4018 induced activation of T cells in the peritoneal cavity (but not the spleen) early after administration. There was evidence of some endogenous T cell activation as indicated by the increased granzyme B expression in T cells in the peritoneal cavity of tumor-bearing mice compared to splenic T cells, and REGN4018 further increased granzyme B expression on both T cell subsets. In addition, REGN4018 resulted in a significant increase in Ki67 expression, a marker of proliferation (Fig. 6D).

**PD-1 blockade enhances antitumor activity of REGN4018**

The PD-1 pathway is a negative regulator of T cell responses. To evaluate whether blockade of the PD-1 pathway can enhance the antitumor effects of REGN4018, we tested antitumor efficacy of REGN4018 alone or in combination with an anti-mouse PD-1 antibody. As shown earlier, REGN4018 induces PD-1 up-regulation on human and cynomolgus T cells in vitro. In addition, a subset of T cells in the peritoneal cavity of mice bearing ID8-VEGF/huMUC16Δ ascites expresses PD-1 (Fig. 7A), and the ID8-VEGF/huMUC16Δ cells express mouse PD-L1 ex vivo (Fig. 7B), validating this model for testing the combination of anti–PD-1 with REGN4018. Intraperitoneal administration of REGN4018 further increased PD-1 on T cells (Fig. 7C). REGN4018 administered alone showed significant antitumor efficacy in this model, and the addition of an anti-mouse PD-1 antibody enhanced the activity of REGN4018; the combination of REGN4018 and anti–PD-1 led to complete tumor clearance in four of the nine mice treated with the combination (Fig. 7D). Blockade of the PD-1 pathway alone did not show any antitumor efficacy alone, as previously described for the ID8 model (26, 30). The ability of PD-1 blockade to enhance antitumor efficacy was also demonstrated in the OVCAR-3 with a suboptimal dose of REGN4018 (fig. S9). These results demonstrate that PD-1 blockade can provide an added benefit to treatment with REGN4018.

**Toxicology study of REGN4018 in cynomolgus monkeys demonstrates a safe toxicity profile**

As shown, REGN4018 cross-reacts with cynomolgus MUC16 and CD3 and induces activation of cynomolgus T cells and potential
cytotoxicity by these T cells. This validates the cynomolgus monkey as a relevant model in which to examine the safety of REGN4018. To determine the safety and tolerability of REGN4018 and characterize the pharmacokinetics, we conducted a multiple-dose toxicity study in cynomolgus monkeys. Six monkeys per sex for each group received weekly administration of REGN4018 for a total of five doses at 0.01, 0.1, or 1 mg/kg, and a control group was administered with vehicle only. At the completion of the dosing period, three animals per sex for each group were euthanized and tissues were examined for microscopic findings, whereas the remaining three animals per sex for each group underwent 12 weeks of treatment-free recovery to assess the reversibility or persistence of any REGN4018-related effects. REGN4018 was well tolerated, and all animals survived to the time of scheduled necropsy. Toxicokinetic analysis demonstrated dose-proportional exposures and linear kinetics across the dose groups, with no gender differences observed (Fig. 8A). Continuous exposure to REGN4018 was not detected in the serum in any animal after recovery week 8. The elimination half-life of REGN4018 was about 10 days.

There were no REGN4018-related clinical observations or any changes in urinalysis parameters, peripheral blood immunophenotyping, food consumption, or body weight during the dosing or recovery periods. REGN4018 administration did not result in any changes in respiratory, neurologic, or cardiovascular safety pharmacology evaluations, including no changes in electrocardiogram parameters. No REGN4018-related changes in organ weight were found, nor were any macroscopic changes noted at either terminal or recovery necropsy. Dose-related, reversible elevations of circulating inflammatory markers [C-reactive protein (CRP) and minimal IL-6] were observed within 1 day after the initial dose of either 1.0 or 0.1 mg/kg, but these elevations were not apparent after subsequent doses (Fig. 8, B and C). In accordance with the minimal increase of serum cytokines, we did not detect changes in T cell numbers in the blood (Fig. 8D), in contrast to what has been described for several CD3 bispecific molecules against hematological tumors (15, 31, 32).

IHC staining for MUC16 was present in expected tissues: pancreas (mesothelium and ductal epithelium), heart, and ovary (Fig. 8E, left), as well as lung (mesothelium and bronchiolar/bronchial epithelium) (fig. S10). REGN4018-related microscopic changes, evaluated by H&E histologic staining, included inflammation (infiltration of white blood cells) and increased mesothelial cell size and cellularity leading to nonadverse thickening of the serosal lining and/or submesothelial connective tissue of multiple thoracic and peritoneal organs. These occurred in animals of all test article–treated groups (REGN4018, ≥0.01 mg/kg per week), with an increase in incidence and/or severity in some tissues from animals that received the highest dose (REGN4018, 1 mg/kg per week). These changes were generally

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**Fig. 5. REGN4018 accumulates in target-bearing tissues including the spleen, LNs, and MUC16-expressing tumors.** WT mice or HuT mice for MUC16 and CD3 were administered with 89Zr-labeled REGN4018 (0.5 mg/kg) or anti-MUC16 parental antibody to track binding in vivo (n = 3 mice per group). (A) Images show analysis from 6 days after dosing. 89Zr-labeled nonbinding control antibody or REGN4018 was administered at 0.5 mg/kg to mice bearing ID8-VEGF/huMUC16Δ tumors implanted 20 days previously. (C) Anti-MUC16 parental antibody (10 mg/kg; second from the right) or CD3-binding control antibody (10 mg/kg; right) was administered at same time as 89Zr-labeled REGN4018 (n = 4 mice per group). Images show analysis from 6 days after dosing. (B and D) Graphs show the percentage of injected dose per gram of tissue (% ID/g), shown as means ± SD.
focal or multifocal in nature, were minimal to slight in severity, and were considered to be on-target for REGN4018, resulting from engagement of MUC16 expressed on serosal epithelial (mesothelial) cells and activation of T cells. The serosal changes were reversed or trended toward reversal at the end of the recovery period (Fig. 8E, right).

DISCUSSION

We described the development and characterization of REGN4018, a human full-length bispecific antibody that targets CD3 and the tumor antigen MUC16. REGN4018 could induce potent cytotoxicity in vitro against MUC16-expressing cells and was not hampered by excess amounts of CA-125, which may be present in patients with ovarian cancer.

The characterization of immuno-oncology clinical candidates is often carried out in immunodeficient mice with engrafted human immune cells. These models can be valuable in demonstrating potency, and indeed, we demonstrated efficacy of REGN4018 in such a model with OVCAR-3 cells. However, immunodeficient models have some limitations. For example, the activation status and proliferation rate of human T cells transferred to an immunodeficient environment may be considerably different to those in an autologous immunocompetent environment. Furthermore, these models do not express human MUC16 in nontumor (normal) tissues and thus do not allow for assessment of effects of REGN4018 on normal MUC16-expressing tissues. Genetically engineered mice offer a model to evaluate antitumor efficacy and on-target, off-tumor effects. In immunocompetent mice genetically HuT for MUC16 and CD3 expression, REGN4018 inhibited growth of two syngeneic tumor models using ID8-VEGF cells transfected with a portion of huMUC16 (a solid tumor model and an ascites model). The xenogenic and syngeneic models complement each other well in their strength and limitations: The xenogenic model demonstrates that REGN4018 can induce human T cells to kill a human tumor line expressing the full-length MUC16 glycoprotein without steric hindrance, whereas the syngeneic model demonstrates T cell activation and killing in the presence of a full immune system with the limitation that only a portion of MUC16 is expressed on the tissues and tumor lines.

Using radiolabeled REGN4018 and immuno-PET imaging, minimal target-mediated binding by the MUC16 arm in HuT mice was observed, perhaps because of very low amounts of the target in normal tissues, limited accessibility of the antibody and T cells to these tissues, or both. In contrast, REGN4018 showed targeting to MUC16-expressing tumors, as well as T cell–rich organs such as spleen and LNs. The addition of an unlabeled anti-CD3 control could block targeting to the spleens and LN, and unlabeled MUC16 parental antibody greatly diminished targeting of REGN4018 to the tumor, demonstrating that much of the targeting to the tumor is driven by the MUC16 arm.

Because cytokine release syndrome is a frequent serious side effect of CD3 bispecific and CAR T cell therapies (33, 34), we monitored serum cytokines in relevant models after treatment with REGN4018. In genetically HuT MUC16/CD3 mice without tumors, no serum cytokine response was evident upon REGN4018 administration. Histopathology examination revealed no inflammation or infiltration into MUC16-expressing tissues in mice after REGN4018 administration at the time examined. The safety profile of REGN4018 was further evaluated in a cynomolgus monkey study. The human MUC16 protein shares 85% amino acid sequence identity with the MUC16 protein of cynomolgus monkey with a 94% sequence identity of the portion of MUC16 containing the last five SEA domains (35, 36). REGN4018 induced only minimal serum cytokines, and
although there was focal induction of inflammation and thickening of the serosal lining in MUC16-expressing tissues suggesting on-target activity, these effects were resolving by the end of the recovery period and consistent with inflammation and increased cellularity indicative of repair. The observed serosal changes were not correlated with any clinical observations, clinical pathology (except inflammatory response), or microscopic changes to the underlying parenchyma. Thus, studies in both genetically HuT mice and cynomolgus monkey show that administration of REGN4018 was well tolerated.

Although immune checkpoint blockade has shown promise in the clinic for a number of cancer types, the clinical responses in ovarian cancer are modest, and thus, combination approaches are likely required (9, 37). REGN4018 potently activates T cells, inducing up-regulation of effector molecules such as granzyme B and increasing PD-1 expression. Tumor cells can express PD-L1, and the induction of IFN-γ and other cytokines in the tumor environment can increase PD-L1 on tumor cells (38, 39). In our murine tumor models, blockade of the PD-1 pathway in combination with REGN4018 enhanced antitumor activity compared to REGN4018 alone, indicating that PD-1 blockade could be a relevant combinatorial approach for our CD3 bispecific. Overall, our results highlight the potent antitumor activity of REGN4018 against MUC16-expressing tumors, even in the presence of soluble CA-125, and also suggest that combination with PD-1 blockade may provide added benefit. These observations support clinical testing of REGN4018 as an immunotherapy in advanced ovarian cancer. A phase 1 trial with REGN4018 as a monotherapy and in combination with anti–PD-1 is currently ongoing (NCT03564340).

**MATERIALS AND METHODS**

**Study design**

The main objective of our study was to determine the antitumor efficacy and safety of REGN4018, a bispecific targeting MUC16 and CD3. The in vivo efficacy was evaluated in both xenogenic and syngeneic mouse models. In the xenogenic model using NSG mice engrafted with human PBMCs and OVCAR-3/Luc cells (sample size of four to six per group), mice were assigned to groups on the basis of T cell engraftment and tumor burden before treatment. For the syngeneic models, sample size was 5 to 12 mice per group. In all studies, all the tumor volume data were plotted. The objective of the nonhuman primate study was to evaluate the safety, pharmacokinetics, and pharmacodynamics of REGN4018. Six monkeys were euthanized at the end of the dosing period (three males and three females), and six were euthanized after an additional 12-week recovery period. Primary data are reported in data file S1.

**Generation of REGN4018**

VelocImmune mice (24, 25), which express human immunoglobulin genes, were immunized to generate CD3- or MUC16-specific antibodies. For MUC16, mice were immunized with a protein containing the last five SEA domains and the C-terminal ectodomain region of human MUC16 (MUC16Δ). For CD3, mice were immunized with the mouse cell line MG-87 expressing human CD3 and later boosted with a cynomolgus monkey CD3ε peptide fused to the Fc portion of mouse IgG2a. Antibodies were screened by binding to OVCAR-3 cells, as well as human and monkey T cells and Jurkat cells. The lead antibodies for MUC16 and CD3ε were assembled with a human IgG4 constant region containing an S228P (serine-to-proline exchange) substitution in the hinge region to minimize half-antibody formation.
Antibodies were produced in Chinese hamster ovary cells and purified using protein A chromatography, as previously described (32).

**Flow cytometry–based cytotoxicity assay using naïve human or cynomolgus monkey effector cells**

To monitor the specific killing of MUC16-bearing target cells by flow cytometry, cell lines expressing various amounts of MUC16
and by reporting the percentage of CD4+ or CD8+ T cells expressing antibody. T cell activation was assessed by incubating cells with the percentage of live target cells were reported for the calculation of adjusted survival. The assay was completed. A total of three studies with four to six mice per group were completed.

Flow cytometry-based cytotoxicity assay using naïve human effector cells in the presence of soluble CA-125 or a MUC16-membrane-proximal construct

We evaluated the ability of REGN4018 to kill MUC16-expressing OVCAR-3 cells in the presence of soluble CA-125 enriched from human ascites (Creative BioMart) or a membrane-proximal construct expressing the last five SEA domains of MUC16 (MUC16Δ). The assay was carried out as described above at an E:T ratio of 4:1 with a fixed concentration of either REGN4018 or CD3-binding control antibody (100 pM) and a serial dilution of either REGN4018 or the CD3-binding control for 72 hours at 37°C. Cells were removed from cell culture plates using trypsin and analyzed by flow cytometry. For cytotoxicity assays using cynomolgus T cells, PBMCs were isolated and activated as previously described (E:T cell ratio, 10:1). For flow cytometry analysis, cells were stained with a fixable far red dead cell stain (Thermo Fisher Scientific). For the assessment of specificity of killing, cells were gated on CellTracker Violet–labeled populations. Percentage of live target cells were reported for the calculation of adjusted survival as follows: adjusted survival = (R1/R2) × 100, where R1 is the percentage of live target cells in the presence of an antibody and R2 is the percentage of live target cells in the absence of a test antibody. T cell activation was assessed by incubating cells with directly conjugated antibodies to CD2, CD4, CD8, and PD-1 by reporting the percentage of CD4+ or CD8+ T cells expressing PD-1 (CD2+).

Xenogenic tumor model

All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol (308) was approved by the Regeneron Pharmaceuticals Institutional Animal Care and Use Committee. NSG (20 to 24 weeks old) mice (the Jackson Laboratory) were injected with 5 × 106 human PBMCs (ReachBio) before 2 × 106 ascites cells from the OVCAR-3/Luc cell line, which were previously passaged in vivo, were administered intraperitoneally (day 0). Mice were checked for T cell engraftment by flow cytometry and then assigned to groups using BLI to ensure similar tumor burden. Mice were treated with REGN4018 or administered an isotype or CD3-binding control to examine antitumor efficacy. In some studies, mice were also treated with anti-CD3 (OKT3) as a positive control for T cell activation. Serum samples were collected for cytokine analysis or to detect drug concentrations at the described time points. Mice were imaged multiple times throughout the study to track tumor burden. For T cell depletion, anti-CD4 (OKT4) and anti-CD8 (OKT8; both Bio X Cell) were administered one time at 50 μg per mouse. A total of three studies with four to six mice per group were completed.

Statistical analysis

Data are presented as means ± SD and means ± SEM as stated in the figure legends. Statistically significant differences were tested using specific tests as indicated in the figure legends. P < 0.05 was considered statistically significant.

SUPPLEMENTARY MATERIALS

Fig S1. REGN4018 lacks antibody-dependent cell-mediated cytotoxicity and complement dependent cytotoxicity functions.

Fig S2. PD-1 and CD25 are up-regulated in response to both REGN4018 and anti-CD3/anti-CD28 stimulation.

Fig S3. REGN4018 binds CA-125 to a much lower degree than anti-MUC16 clone 3A5.

Fig S4. REGN4018 did not induce any weight loss in animals with detectable drug concentrations.

Fig S5. MUC16 expression is maintained in vivo after REGN4018 treatment.

Fig S6. BLI at baseline correlates with complete tumor clearance.

Fig S7. Human T cells are maintained after transfer of human PBMCs and can respond to antigen.

Fig S8. Antitumor efficacy and cytokine induction by REGN4018 require T cells and MUC16-expressing OVCAR-3/Luc cells.

Fig S9. PD-1 blockade enhances efficacy of suboptimal REGN4018 in the xenogenic OVCAR-3 model.

Fig S10. MUC16 expression in the lung from cynomolgus monkey.

Data file S1. Primary data.

REFERENCES AND NOTES


Competing interests: Regeneron Pharmaceuticals Inc. is developing REGN4018 as a clinical compound. All authors are employees of Regeneron Pharmaceuticals Inc. Regeneron Pharmaceuticals Inc. has filed patent applications related to this work for REGN4018, and A.C., L.H., M.P.K., E.S., and J.R.K. are inventors on the patent application (application no. 20180118848). Data and materials availability: All data associated with this study are present in the paper or the Supplementary Materials. Regeneron materials described in this manuscript may be made available to qualified, academic, noncommercial researchers through a material transfer agreement upon request at https://regeneron.envisionpharma.com/vt_regeneron/. For questions about how Regeneron shares materials, use the email address (preclinical.collaborations@regeneron.com).

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A Mucin 16 bispecific T cell–engaging antibody for the treatment of ovarian cancer

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Opening up options in ovarian cancer

The glycoprotein Mucin 16 (MUC16) has been previously investigated as a targetable tumor antigen. Crawford et al. now report on a bispecific antibody that binds CD3 and MUC16, which could potentially be used in ovarian cancer treatment. The antibody induced T cell activation and ovarian cancer cells killing both in vitro and in mouse models. This antibody worked well with checkpoint blockade and was shown to be safe when administered to nonhuman primates. A clinical trial using this antibody is already underway.